RESEARCH PAPER

Attenuated accumulation of jasmonates modifies stomatal responses to water deficit

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Abstract

To determine whether drought-induced root jasmonate [jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile)] accumulation affected shoot responses to drying soil, near-isogenic wild-type (WT) tomato (Solanum lycopersicum cv. Castlemart) and the def-1 mutant (which fails to accumulate jasmonates during water deficit) were self- and reciprocally grafted. Rootstock hydraulic conductance was entirely rootstock dependent and significantly lower in def-1, yet def-1 scions maintained a higher leaf water potential as the soil dried due to their lower stomatal conductance (gs). Stomatal sensitivity to drying soil (the slope of gs versus soil water content) was low in def-1 self-grafts but was normalized by grafting onto WT rootstocks. Although soil drying increased 12-oxo-phytodienoic acid (OPDA; a JA precursor and putative antitranspirant) concentrations in def-1 scions, foliar JA accumulation was negligible and foliar ABA accumulation reduced compared with WT scions. A WT rootstock increased drought-induced ABA and JA accumulation in def-1 scions, but decreased OPDA accumulation. Xylem-borne jasmonates were biologically active, since supplying exogenous JA via the transpiration stream to detached leaves decreased transpiration of WT seedlings but had the opposite effect in def-1. Thus foliar accumulation of both ABA and JA at WT levels is required for both maximum (well-watered) gs and stomatal sensitivity to drying soil.

Keywords: Drought stress, JA, ABA, grafting, Solanum lycopersicum.

Introduction

Jasmonic acid (JA) and other members of the oxylipin pathway such as 12-oxo-phytodienoic acid (OPDA) and the bioactive jasmonic acid-isoleucine (JA-Ile; Katsir et al., 2008; Fonseca et al., 2009) have been associated with tolerance to biotic stresses (Wasternack and Hause, 2013) by mediating defence against herbivores and necrotrophic pathogens. JA and its derived molecules (mainly JA-Ile as the bioactive interactor with the COI1 receptor) are known as jasmonates, a subgroup of oxylipins derived from OPDA also associated with the systemic response to physical stresses such as wounding (Sun et al., 2011). Nevertheless, they can also be involved in tolerance to other abiotic stresses such as drought and salinity (Cheng et al., 2013; Alam et al., 2014; Savchenko et al., 2014; Kazan, 2015). Water deficit increases tissue JA concentrations in several species (Creelman and Mullet, 1995; Mahouachi et al., 2007; Brossa et al., 2011; Chen et al., 2016), as well as JA-Ile (De Ollas et al., 2015), while mutations in several steps of the JA biosynthesis and signalling pathways alter tolerance to water deficit (Harb et al., 2010; Grebner et al., 2013; Riemann et al., 2015). Although JA and abscisic acid (ABA) seem to influence each other’s biosynthesis (Bandurska et al., 2003; Kim et al., 2009; De Ollas et al., 2013; Fragoso et al., 2014), few studies have assessed...
their relative importance in mediating physiological responses to water deficit.

Although ABA plays a central role in regulating stomatal closure in response to soil drying (Raghavendra et al., 2010; Lee and Luan, 2012), other phytohormones can also influence this response. Exogenous application of either ABA or methyl jasmonate (MeJA) elicited similar stomatal closure using the same second messengers (Munemasa et al., 2011). The oxylipin OPDA is claimed to promote stomatal closure more effectively and independently from JA and in co-operation with ABA (Savchenko et al., 2014). JA/JA-Ile-dependent stomatal regulation may also be important since applying ABA or MeJa to the Arabidopsis jarl-1 mutant (which is defective in conjugating isoleucine to JA) elicited less stomatal closure than in wild-type (WT) plants (Suhita et al., 2004). Interestingly, the Arabidopsis aos (JA/JA-Ile and OPDA deficient) and the opr3 (deficient in JA/JA-Ile but not OPDA) mutants had higher and lower stomatal aperture than WT plants, respectively (Savchenko et al., 2014). Thus various oxylipins and ABA may be involved in regulating stomatal responses of plants in drying soil.

While these studies explored stomatal responses of isolated epidermal peels (reviewed in De Ollas and Dodd, 2016), few studies have examined JA/JA-Ile effects on whole-plant transpiration. While leaf scorching increased JA concentration concurrent with stomatal closure in distal leaves (Hevāčková et al., 2006), withholding water from the WT and the Arabidopsis aos mutant (JA/JA-Ile and OPDA deficient) elicited similar stomatal closure (Brossa et al., 2011). Similar drought-induced stomatal closure in genotypes varying in oxylipin status is consistent with a model where stomatal behaviour is primarily determined by plant hydraulics (Tardieu, 2016). In this context, exogenous application of ABA (reviewed in Dodd, 2013) or MeJA (Sánchez-Romera et al., 2014) increased root hydraulic conductivity (Lr) along with gene expression of several aquaporins (Sánchez-Romera et al., 2014). Moreover, a tomato (Solanum lycopersicum) mutant (def-1) defective in stress-induced JA biosynthesis had lower Lr, which was restored by MeJA treatment (Sánchez-Romera et al., 2014). Although phytohormone-mediated variation in Lr may influence leaf water relations, whether this regulates stomatal responses to soil drying is less clear. Reciprocal grafting with different root and scion genotypes determined the role of long-distance JA/JA-Ile signalling in wound responses (Fragoso et al., 2014), but the impact of variation in root and shoot jasmonates on plant water relations responses to soil drying has not been assessed.

Since both ABA and JA can regulate both Lr and stomatal conductance (gs), JA may alter physiological responses to drying soil. Initially, ungrafted plants of two tomato mutants compromised in the oxylipin pathway [spr2, constitutively deficient in the biosynthesis of OPDA, JA, and the bioactive JA-Ile (Li et al., 2003); and def-1, with diminished stress-induced JA/JA-Ile accumulation (Howe et al., 1996)] were grown under water deficit to characterize root and shoot phytohormonal responses. Then, to better understand the involvement of both scion- and root-sourced jasmonates in physiological responses to water deficit, self- and reciprocally grafted WT and def-1 mutant (with attenuated JA accumulation in drying soil) plants were grown in well-watered and drying soil. Physiological responses such as whole-plant transpiration, leaf and root water potential, Lr, and gs were correlated with leaf and xylem sap ABA and oxylipin (OPDA and JA/JA-Ile) concentrations. To determine whether xylem-supplied phytohormones regulate stomatal responses, detached leaves were supplied with JA or ABA via the transpiration stream (Zhang and Davies, 1991). We tested the hypothesis that jasmonates can regulate transpiration via local (foliar jasmonates accumulation) but also long-distance (root to shoot transport through the transpiration stream) processes.

Materials and methods

Plant material

To characterize jasmonate effects on plant responses to water deficit, two tomato (S. lycopersicum) mutants (def-1 and spr2) were grown under well-watered and water-limited conditions. The (unidentified) def-1 mutation (originally described as JL5; Howe et al., 1996) prevents JA (and hence JA/Ile) accumulation under wounding and biotic stress, but basal JA/JA-Ile concentrations are similar to those of its near-isogenic line Castenmatt. This genetic lesion is not related to any identified biosynthetic gene and this mutant is not compromised in OPDA biosynthesis (Howe et al., 2000). Thus def-1 has attenuated stress-induced JA/JA-Ile accumulation.

In the spr2 (suppressor of prosystemin-mediated responses 2; Howe et al., 1996) mutant, the lack of a chloroplast fatty acid desaturase in the octadecanoid pathway compromises constitutive JA biosynthesis, severely decreasing leaf (Li et al., 2003) and root (Tejeda-Santorius et al., 2008) JA levels. Wound-induced JA biosynthesis is also impaired (Li et al., 2003) as is OPDA biosynthesis (Wasternack et al., 2012). Thus spr2 is more generally deficient in both jasmonates and OPDA biosynthesis.

Experiments with ungrafted plants

Seeds of the WT, spr2, and def-1 were sown individually in 1 cm diameter plastic pots filled with a mixture of peat/perlite (80:20, v/v) and covered with black plastic to ensure high humidity and darkness to promote germination. After 4–6 d, the plastic was removed to prevent seedling etiolation. After a further week, seedlings were potted into individual 216 cm³ pots and watered daily for a further 2 weeks, then transplanted to 4000 cm³ pots filled with the same substrate. After another week under well-watered conditions, pots were covered with plastic bags to limit soil evaporation. Randomly selected plants of each genotype were divided into well-watered (daily replacement of evapotranspiration) and water deficit [soil water content (SWC) allowed to drop to <0.2–0.15 cm in height (750 cm volume), with stainless steel mesh (0.7 mm deficit | soil water content (SWC) allowed to drop to <0.2–0.15 cm³ before re-watering] groups. After 5 d of soil drying when all genotypes were at a similar SWC, whole-plant transpiration and water potential of the youngest fully expanded leaf (ΨL,defirst|soilwatercontent(SWC)allowedtodropto<0.2–0.15cm³beforerewatering)groups.After5dofsoildryingwhenallgenotypes were measured as described above, with xylem sap collected from this leaf. Prior to measuring ΨL, the next youngest leaf was excised and placed in liquid N₂. Then the roots were carefully washed from the substrate, and placed in liquid N₂.

Experiments with grafted plants

Seeds of WT and def-1 tomato were sown individually in 1 cm diameter plastic pots filled with Levinton M3 compost (Scotts Company Ltd, Ipswich, UK) and covered with black plastic to ensure high humidity and darkness to promote germination. After 4–6 d, the plastic was removed to prevent seedling etiolation. After a further week, seedlings were potted into individual 500 cm³ pots and watered daily for a further 2 weeks prior to grafting. Graft unions were established just below the cotyledonary node as previously described (Dodd et al., 2009). Two weeks after grafting, plants were transplanted to pots containing an organic loam (John Innes No. 2, UK), for which a moisture release curve has been previously published (Dodd et al., 2010). Pots were cylinders, 6.5 cm in diameter and 23 cm in height (750 cm³ volume), with stainless steel mesh (0.7 mm...
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Three plants of each graft combination were randomly selected to measure root hydraulic conductivity. After shoot removal, a series of overpressures (from 0.1 MPa to 0.6 MPa in 0.1 MPa increments) were applied and the sap flow rate determined at each pressure. Roots were washed from the pots and kept moistened with wet paper, before scanning (Epson perfection v700 photo), with the scanner connected to a computer running the WinRHIZO™ software (Regent Instruments, Canada). Root hydraulic conductivity quantifies root permeability to the flow of water, by applying increasing pneumatic pressure to the root zone. Applied pressure and water flow are linearly correlated, with the slope of the relationship defined as the root hydraulic conductance (when data are normalized to root surface area).

**Detected leaf transpiration assays**

Seeds of the *dof*-*1* and *spr*-*2* mutants and their corresponding WT (cv. Castlemart) were sown as described above, and then potted into individual 350 cm³ pots filled with a mixture of peat/perlite (80/20, v/v). These were placed in a controlled-environment chamber with a day/night temperature of 25/16°C and a 16 h photoperiod (07.00–23.00 h), and watered every 2–3 d. Day/night relative humidity was 42/54%, CO₂ concentration was 450/380 ppm, and light intensity at plant height between 400 µmol m⁻² s⁻¹ and 500 µmol m⁻² s⁻¹. PPFD. Plants were kept in those conditions for a further 3 weeks prior to the experiments.

Well-watered plants (6 weeks old, irrigated to drained capacity) were kept in the dark overnight. A razor blade severed fully expanded leaves at the petiole–stem junction, which were then recut (5 mm from the initial excision site) under deionized water to prevent xylem embolism. The leaves were immediately transferred to a 20 ml glass vial containing 15 ml of artificial xylem sap (the same composition as in Dodd et al., 2003), and placed in a dark room for 2 h. The top of the glass vial was sealed by parafilm, with a small hole to insert the petiole in the artificial xylem sap (whilst reducing evaporative losses). After 2 h, all leaves were randomly transferred to glass vials containing 15 ml of artificial xylem sap with different ABA or JA concentrations (0, 10, 100, and 1000 nM). After these preliminary dose–response curves, further experiments used 1000 nM as the minimum concentration of JA that significantly decreased transpiration of WT plants.

Leaves were then randomized within the controlled-environment room with conditions described in the previous paragraph. Each vial was weighed initially by a four-point analytical balance, then re-weighed hourly for 5 h. Leaves that showed any (wilting) symptoms of embolism (<10% of the total) were discarded. Finally, leaf area was recorded using a scanner and the Easy Leaf Area software to normalize the transpiration rate for leaf area. After the assay, petioles were gently rinsed with distilled water to remove any of the feeding solution, the leaf was placed in a pressure chamber, and xylem sap (100 µl per sample) was collected at a fixed overpressure (0.1–0.2 MPa) above balancing pressure. Leaves were then frozen in liquid N₂ to analyse the hormone concentrations.

To determine possible wounding-related hormonal changes caused by excising leaves for use in the transpiration assay, comparable leaves were collected from intact plants grown under the same conditions, both before (pre-excision control) and after the transpiration assay (post-excision control). These leaves were immediately placed in the pressure chamber to collect xylem sap as described above, then frozen in liquid N₂ to analyse hormone concentration.

**Phytohormone quantification**

ABA, JA, OPDA, and JA-Ile were extracted and analysed essentially as previously described (De Ollas et al., 2013) with slight modifications. Briefly, 0.2 g of dry plant material was extracted in 2 ml of distilled H₂O after spiking with 25 µl of a 2 mg l⁻¹ solution of d⁴-ABA and dihydrojasmonic acid (DHJA) as internal standards. After centrifugation (10 000 g at 4°C), supernatants were recovered and the pH was adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 ml of di-ethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (SpeedVac, Jouan, Saint Herblain Cedex, France). The dry residue was then re-suspended in a 9:1 H₂O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a UPLC system (waters Acuity SDS, Waters Corp., Milford, MA, USA) interfaced to a TOQ triple quadrupole (Micromass Ltd, Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. Xylem sap (75 µl) was spiked with 10 µl of internal standard solution and diluted with deionized water to a total volume of 200 µl; the solution was filtered and injected into the UPLC system using the same conditions as used for tissue extraction.
Separations were carried out on a Gravity C18 column (50 × 2.1 mm, 1.8 μm, Macherey–Nagel GmbH, Germany) using a linear gradient of MeOH and H₂O supplemented with 0.1% acetic acid at a flow rate of 300 μl min⁻¹. Transitions for ABA/d-ABA (263>153/269>139), JA/ DHJA (209>59/211>59), OPDA (291>165), and JA-Ile (322>130) were monitored in negative ionization mode. Quantitation of plant hormones was achieved by external calibration with known amounts of pure standards using Masslynx v4.1 software.

Stomatal density and size analysis
Epidermal imprints were taken from fully expanded leaves following a similar protocol to that of Scalschi et al. (2015). Leaflets were placed on glass slides with the abaxial epidermis in contact with dental resin. For stomatal analysis, images of randomly selected regions were taken using a Leica IRB microscope equipped with a Leica DC300F camera (Leica Microsystems CMS GmbH, Wetzlar, Germany). Stomatal number and size of at least 30 random sections per genotype were calculated with the ImageJ software. Size or stomatal area was calculated assuming the area of an ellipse.

Statistical analysis
In ungrafted plants (Table 1) and the detached leaf experiments (Figs 8, 9), genotype and treatment effects (and their interaction) were determined using two-way ANOVA, with Duncan’s HSD (P<0.05) used to determine significant differences between genotype/treatment combinations. In grafted plants, rootstock and scion effects (and their interaction) were determined using two-way ANOVA, with significant effects of graft combinations on the relationships between soil and plant variables determined by analysis of covariance (ANCOVA; e.g. Figs 2–7). Differences in detached leaf transpiration and foliar phytohormone concentrations (Figs 8, 9) between genotype/treatment combinations were determined using one-way ANOVA, with significant (P<0.05) differences determined using Duncan’s HSD.

Table 1. Whole-plant transpiration, leaf area, leaf water potential, and ABA, OPDA, JA, and JA-Ile concentrations in leaves, xylem sap, and roots of the wild type (WT), def-1, and spr2 under well-watered (WW) and water deficit (WD) conditions

|                  | Whole type (WT) | def-1 | spr2 | P-values from two-way ANOVA |
|------------------|----------------|-------|------|-----------------------------|
|                  | WW            | WD    | WW   | WD             | Genotype | Treatment | G×T   |
| Whole-plant transpiration (mmol m⁻² s⁻¹) | 2.18 ± 0.06 a | 0.70 ± 0.06 c | 1.41 ± 0.05 b | 0.45 ± 0.12 c | 2.50 ± 0.15 a | 0.77 ± 0.45 c | <0.001 | <0.001 | <0.006 |
| Leaf area (cm²) | 430 ± 32 a | 416 ± 25 a | 378 ± 24 a | 385 ± 15 a | 330 ± 36 b | 325 ± 19 b | 0.003 | 0.771 | 0.802 |
| Leaf water potential (MPa) | 243 ± 9 b | 547 ± 30 a | 252 ± 5 b | 602 ± 59 a | 228 ± 59 a | 660 ± 55 a | 0.301 | <0.001 | 0.234 |
| Leaf [ABA] (ng g⁻¹ DW) | 27 ± 4 cd | 34 ± 5 c | 54 ± 2 b | 83 ± 7 a | 14 ± 2 de | 11 ± 5 e | 0.007 | <0.001 | 0.009 |
| Leaf [OPDA] (ng g⁻¹ DW) | 13 ± 1 bc | 49 ± 3 a | 15.5 ± 1.6 b | 17 ± 2 b | 5.1 ± 0.5 d | 8.1 ± 0.9 cd | <0.001 | <0.001 | <0.001 |
| Leaf [JA] (ng g⁻¹ DW) | 2.7 ± 0.2 b | 15 ± 1 a | 2.1 ± 0.1 bc | 3.5 ± 0.6 b | 0.4 ± 0.1 c | 0.62 ± 0.01 c | <0.001 | <0.001 | <0.001 |
| Leaf [JA-Ile] (ng g⁻¹ DW) | 189 ± 15 c | 2775 ± 386 a | 193 ± 15 c | 2178 ± 284 ab | 197 ± 32 c | 1894 ± 409 b | 0.253 | <0.001 | 0.242 |
| Xylem sap [ABA] (nM) | 58 ± 7 cd | 85 ± 7 bc | 96 ± 78 b | 164 ± 14 a | 30 ± 13 d | 54 ± 7 d | <0.001 | <0.001 | 0.081 |
| Xylem sap [OPDA] (nM) | 95 ± 8 cd | 385 ± 20 a | 119 ± 17 b | 152 ± 21 b | 31 ± 5 de | 58 ± 11 de | <0.001 | <0.001 | <0.001 |
| Xylem sap [JA] (nM) | 1.9 ± 0.2 bc | 3.7 ± 0.7 a | 1.4 ± 0.2 c | 2.5 ± 0.2 b | 0.11 ± 0.04 d | 1.0 ± 0.2 cd | <0.001 | <0.001 | 0.328 |
| Xylem sap [JA-Ile] (nM) | 247 ± 17 c | 1825 ± 320 a | 238 ± 320 a | 2422 ± 227 a | 240 ± 18 c | 2025 ± 240 a | 0.333 | <0.001 | 0.314 |
| Root [ABA] (ng g⁻¹ DW) | 26 ± 3 d | 57 ± 3 b | 46 ± 3 c | 68 ± 7 a | 9 ± 1 e | 16 ± 2 de | <0.001 | <0.001 | 0.016 |
| Root [OPDA] (ng g⁻¹ DW) | 86 ± 13 b | 209 ± 29 a | 62 ± 3 bc | 40 ± 4 cd | 8 ± 2 d | 13 ± 3 de | <0.001 | 0.005 | <0.001 |
| Root [JA] (ng g⁻¹ DW) | 13 ± 2 b | 23 ± 3 a | 8 ± 1 bc | 11 ± 1 b | 1.3 ± 0.6 d | 3.6 ± 0.6 cd | <0.001 | 0.002 | 0.074 |

Values are the mean ±SE of four replicates, while different letters denote significant (P<0.05) differences across genotypes and treatments. The table summarizes significance (P-values) of genotype (G), treatment (T), and their interaction (G×T) after ANOVA.
spr2 plants (see Supplementary Fig. S1 at JXB online). Under WW conditions, all genotypes had a similar leaf water potential (Ψ_leaf), but, under WD conditions, Ψ_leaf of def-1 plants was higher than in the WT and spr2 (–0.7, –0.97, and –1.08 MPa, respectively). These differences in shoot water relations were not due to differences in SWC (data not shown).

WT, def-1, and spr2 plants had similar tissue ABA concentrations under both WW and WD conditions. Water deficit increased root and leaf ABA concentrations by 8.7- and 2.5-fold, respectively (averaged across all genotypes). Although leaf xylem sap ABA concentrations under WW conditions did not differ between genotypes, under WD conditions xylem ABA concentrations of def-1 and spr2 plants were 22% and 32% lower than that of WT plants, respectively. This genetic variation in ABA status did not affect transpiration.

OPDA concentrations differed significantly between treatments and genotypes. Under WW conditions, root OPDA concentrations of def-1 and spr2 were 70% higher and 70%
lower than those of the WT, respectively. Water deficit stimulated root OPDA accumulation by 1.8-fold (averaged across all genotypes). Likewise, water deficit stimulated foliar OPDA accumulation in WT and def-1 plants, but not in spr2 plants. Xylem sap OPDA concentrations showed similar responses, which were 78% higher in def-1 but 42% lower in spr2 (averaged across water treatments). Water deficit increased xylem OPDA concentrations by 65% (averaged across genotypes).

Under WW conditions, the WT and def-1 had similar tissue and xylem sap JA concentrations, whereas spr2 had significantly lower (by 60–80%) JA concentrations than the WT. In WT plants, water deficit increased root, leaf xylem sap, and leaf JA concentrations by 2.4-, 4-, and 3.8-fold, respectively. However, water deficit did not significantly increase JA accumulation in def-1 and spr2 plants. JA-Ile concentrations showed similar genotypic and treatment differences to JA, except that xylem JA-Ile concentration significantly increased in all genotypes, but with lower absolute values in both mutants.

Since def-1 plants had similar leaf area but different transpiration responses to WT plants (Table 1), the role of differential oxylipin accumulation and root and shoot regulation of plant water relations was investigated by reciprocally grafting WT and def-1 plants. Moreover, these graft combinations allowed the physiological effects of attenuated water deficit-induced JA accumulation to be assessed independently of constitutively low oxylipin status.

**Grafted plants**

Under WW conditions, hormonal imbalance of def-1 self-grafts decreased whole-plant transpiration by 43% compared with WT self-grafts (Fig. 1). Both reciprocal graft combinations had intermediate, similar whole-plant transpiration rates, which were 25% lower than that of WT self-grafts. Thus hormonal imbalance in either scion or rootstock significantly (P<0.001 for rootstock and scion main effects) decreased whole-plant transpiration.

Stomatal conductance (g_s) declined linearly with SWC in all graft combinations (Fig. 2A, B). Sensitivity to soil drying (defined as the slope of g_s versus SWC) was generally similar in all graft combinations (except def-1 self-grafts) once g_s values were normalized according to the maximum g_s of each graft combination (Fig. 2C). Although g_s of def-1 self-grafts was less sensitive to drying soil, all graft combinations had similar absolute g_s values when the soil profile was depleted of soil moisture. Thus the def-1 genotype as either scion or rootstock significantly decreased maximum g_s, but only def-1 self-grafts had diminished stomatal sensitivity to drying soil.

Under WW conditions, all graft combinations had similar values of leaf water potential (Ψ_leaf ranged between −0.4 MPa and −0.55 MPa (Fig. 3A)) and root water potential (Ψ_root ranged between −0.01 (with some plants showing spontaneous exudation with no applied pressure) and −0.19 MPa). Root water potential declined exponentially as the soil dried, but similarly in all graft combinations (Fig. 3B). Leaf water potential declined linearly with SWC (Fig. 3A), but more so in WT scions (significant scion×SWC interaction). Taken together, this implies that drought-induced stomatal closure in def-1 scions (Fig. 2B) was more effective at regulating Ψ_leaf than in WT scions (Fig. 3A).

Stomatal conductance decreased linearly with Ψ_leaf in WT scions (Fig. 4A), but exponentially in def-1 scions (Fig. 4B). The rootstock had no impact on the sensitivity of g_s to Ψ_leaf in either def-1 or WT scions (once g_s values were normalized according to the maximum g_s of each graft combination).

Root hydraulic conductance (L_pr), calculated as the slope of sap flow from de-topped root systems versus applied pressures and normalized to root surface area, was entirely rootstock dependent (Fig 5A). L_pr of WT rootstocks was approximately double that of def-1 rootstocks (Fig. 5B).

Foliar ABA accumulation increased with soil drying in all graft combinations (Fig. 6A), but WT scions accumulated significantly more ABA in leaves of plants at low SWC (significant scion×SWC interaction). When exposed to drying soil, ABA accumulation in WT scions was 24% and 50% higher...
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than in the def-1/WT (scion/rootstock) and def-1/def-1 graft combinations, respectively. Although a def-1 rootstock had no significant impact on drought-induced ABA accumulation in WT scions, a WT rootstock enhanced ABA accumulation in def-1 scions (significant scion×rootstock interaction). Stomatal closure was correlated with foliar ABA accumulation (Fig. 7A), but was more sensitive in def-1 scions, which accumulated half as much ABA at maximal stomatal closure.

Foliar JA concentration also increased with soil drying in all graft combinations except def-1 self-grafts (Fig. 6B). JA accumulation in WT scions was double that of the def-1/WT graft combination, while def-1 self-grafts did not accumulate JA when exposed to drying soil. Rootstock genotype did not significantly affect JA accumulation in WT scions, but a WT rootstock significantly increased drought-induced JA accumulation in def-1 scions (significant rootstock×SWC interaction). JA concentration increased with stomatal closure in WT scions (Fig. 7B), whereas this trend neared significance (P=0.065) in the def-1/WT graft combination. Although absolute JA/JA-Ile concentrations were ~5-fold lower in all graft combinations compared with the experiments with ungrafted plants (cf. Table 1), these were probably due to differences in analytical (instrument) sensitivity, and both experiments showed similar treatment and genotypic relative differences.

Soil drying induced a similar pattern of JA-Ile accumulation in the graft combinations as JA (Fig. 6D). JA-Ile accumulation in WT scions was 38% higher than that of the def-1/WT graft combination, while def-1 self-grafts did not accumulate JA-Ile...
when exposed to drying soil. However, rootstock genotype did not affect JA-Ile accumulation in WT or def-1 scions. Only in WT scions did JA-Ile concentrations increase with stomatal closure (Fig. 7D).

Whereas soil drying induced only limited (36% increase) foliar OPDA accumulation in WT self-grafts and had no effect in WT/def-1 plants (Fig. 6C), soil drying substantially increased OPDA concentrations of def-1 scions (significant scion×SWC interaction). WT rootstocks decreased OPDA accumulation in def-1 scions (significant scion×rootstock×SWC interaction), such that foliar OPDA concentrations were 44% lower than in def-1 self-grafts. Stomatal conductance was not correlated with OPDA concentration in WT scions, but leaf OPDA concentrations increased with stomatal closure in def-1 scions (Fig. 7C).

Water deficit decreased sap flow of all graft combinations (Table 2); however, plants with a def-1 scion had significantly lower flow rates than plants with a WT scion (the significance, P-values, of yhr flow rate and hormone delivery are summarized in Table 3). All graft combinations had similar root xylem ABA concentrations under both WW and WD conditions, with water deficit increasing xylem ABA concentrations by 11.5-fold from 37 nM to 426 nM (averaged across graft combinations). Xylem JA concentrations were similar in all graft combinations under WW conditions (48 nM); however, water deficit significantly increased JA concentrations of graft combinations with a WT rootstock (to 1030 nM). JA-Ile followed a similar trend, with low concentrations under WW conditions but higher JA-Ile concentrations when graft combinations with a WT rootstock dried the soil.

Hormone delivery from the root system was quantified by multiplying hormone concentration and pressure-induced xylem flow (Table 2). Although water deficit increased ABA delivery by an order of magnitude, all graft combinations had similar ABA delivery. In contrast, both treatment and root/shoot genotype affected JA delivery. Although all graft combinations had similar root JA export under WW conditions, water deficit increased root JA export of WT self-grafts, both reciprocal grafts, and def-1 self-grafts by 7-, 5-, and 2.6-fold, respectively. Root JA-Ile export followed a similar pattern. Thus water deficit always increased root JA/JA-Ile export, even if it had no measurable effect on foliar JA/JA-Ile concentrations (as in def-1 self-grafts).

Detached leaf transpiration assays

To determine whether xylem-supplied phytohormones could regulate transpiration, ABA or JA were supplied via the transpiration stream (to emulate root to shoot hormone delivery) to detached WT, def-1, and spr2 leaves. Detached leaf transpiration was more sensitive to xylem-supplied ABA than...
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**Fig. 7.** Stomatal conductance versus foliar ABA (A), JA (B), OPDA (C), and JA-Ile (D) concentrations in wild-type (WT) self-grafts (filled circles), def-1 self-grafts (open triangles), and reciprocal graft (scion/rootstock) combinations (WT/def-1, open circles; and def-1/WT, filled triangles). Correlations between hormone concentration and stomatal conductance reported ($r^2$ and $P$-values) for WT scions (dashed lines), def-1 scions (dotted lines), and def-1/WT plants (plain line). The tables summarize the significance ($P$-values) of hormones, scion, rootstock, and their interactions after ANOVA.

**Table 2.** Xylem sap flow rate, root xylem sap hormone concentrations (ABA/JA/JA-Ile), and hormone delivery (the product of sap flow rate and hormone concentration) under well-watered (WW) and water deficit (WD) conditions in the wild type (WT/WT) and def-1 (def-1/def-1) self-grafts and reciprocal grafts (WT/def-1, def-1/WT)

|                | WT/WT | WT/def-1 | def-1/WT | def-1/def-1 |
|----------------|-------|----------|----------|-------------|
| Flow (nmol s$^{-1}$)$\times 10^3$ | 4.8 ± 0.4 a | 1.8 ± 0.3 d | 3.4 ± 0.9 b | 1.6 ± 0.3 de | 3.0 ± 0.5 b | 1.4 ± 0.2 ef | 2.6 ± 0.4 c | 1.1 ± 0.1 f |
| [ABA] (nM)     | 44 ± 5 b | 427 ± 35 a | 34 ± 4 b | 378 ± 44 a | 31 ± 2 b | 475 ± 52 a | 38 ± 2 b | 538 ± 48 a |
| [JA] (nM)      | 52 ± 11 bc | 1047 ± 154 a | 57 ± 9 bc | 598 ± 68 b | 43 ± 27 bc | 1014 ± 69 a | 42 ± 5 c | 426 ± 42 bc |
| [JA-Ile] (nM)  | 4.1 ± 0.2 c | 73 ± 9 a | 2.7 ± 0.9 c | 45 ± 13 ab | 3.9 ± 0.6 c | 66 ± 15 a | 6.4 ± 1.4 c | 26 ± 2 c |
| ABA delivery (nmol s$^{-1}$)$\times 10^3$ | 3.8 ± 0.2 b | 14.2 ± 0.1 a | 2.1 ± 0.2 b | 11 ± 1 a | 1.7 ± 0.2 b | 11.9 ± 0.2 a | 1.4 ± 0.1 b | 10.7 ± 0.9 a |
| JA delivery (nmol s$^{-1}$)$\times 10^5$ | 4.5 ± 0.6 d | 35 ± 5 a | 3.5 ± 0.9 d | 18 ± 3 b | 5.4 ± 0.7 d | 25 ± 4 b | 4.0 ± 0.4 d | 8.5 ± 2.3 c |
| JA delivery (nmol s$^{-1}$)$\times 10^5$ | 0.3 ± 0.1 b | 2.4 ± 0.4 a | 0.2 ± 0.1 b | 1.3 ± 0.5 a | 0.2 ± 0.1 b | 1.6 ± 0.4 a | 0.2 ± 0.1 b | 0.5 ± 0.2 b |

Values are the mean ± SE of four replicates. Letters denote significant ($P<0.05$) differences across genotype and treatment after a post-hoc (Duncan’s HSD).

xylem-supplied JA, with significant reductions at 100 nM and 1000 nM, respectively (Supplementary Fig. S2). As in whole plant measurements, the transpiration rate of detached def-1 leaves was (21%) lower than that of WT leaves when supplied with an artificial xylem solution, while spr2 transpiration was significantly (9.6%) higher (Fig. 8). Xylem-supplied JA (1000 nM) decreased transpiration of WT and spr2 leaves by 16% and 24%, respectively, but increased transpiration of def-1 leaves by 23%. Xylem-supplied ABA (1000 nM) decreased transpiration in all genotypes, but more effectively in WT and spr2 (50% reduction) than in def-1 (26% reduction).

To determine whether xylem-supplied ABA or JA influenced synthesis or metabolism of each other, foliar (Fig. 9) and xylem sap (Supplementary Fig. S3) phytohormone concentrations were quantified at the end of the transpiration assay, after supplying artificial xylem sap via the transpiration stream for 5 h. All three genotypes had similar ABA and JA/JA-Ile concentrations to freshly harvested leaves (pre- and post-excision
Table 3. A summary of the significance (P-values) of rootstock genotype (R), scion genotype (S), treatment (T), and interactions on sap flow and hormone delivery after ANOVA

| Factor          | Xylem sap VS ABA | Xylem sap VS JA |
|-----------------|------------------|-----------------|
| Genotype        | <0.001           | 0.537           |
| Treatment       | <0.001           | <0.001          |
| G x T           | <0.001           | <0.001          |

Flow | ABA delivery | JA delivery | JA-Ile delivery |
-----|--------------|-------------|-----------------|
Treatment | <0.001 | <0.001 | <0.001 |
Root     | <0.001 | 0.042 | <0.001 |
Shoot    | <0.001 | 0.062 | 0.004 |
T x R    | 0.05  | 0.68  | <0.001 |
T x S    | 0.06  | 0.2   | 0.026 |
R x S    | 0.326 | 0.45  | 0.44 |
T x R x S| 0.804 | 0.26  | 0.51 |

JA-Ile concentrations by 5.3-fold, averaged across both genotypes (Fig. 9G). Taken together, xylem-supplied ABA significantly increased JA-Ile concentrations in WT and mutant genotypes, while significantly decreasing OPDA concentrations in def-1.

Xylem-supplied JA had no effect on leaf ABA concentration in all genotypes (Fig. 9A), but substantially increased JA and JA-Ile concentrations (Fig. 9E, G). These increases were much greater in WT plants (5.5-fold averaging both JA and JA-Ile) than in def-1 (3.4-fold) and spr2 (4-fold). Xylem-supplied JA had no effect on leaf OPDA concentrations in WT and spr2 plants (Fig. 9C), but decreased OPDA concentrations of detached def-1 leaves by 51% compared with plants supplied with artificial xylem sap (Fig. 9C, D). Genotypic variation in oxylipin status in leaves supplied with JA via the transpiration stream was not explained by genotypic differences in JA uptake.

Taken together, supplying ABA or JA via the transpiration stream to detached leaves had the intended effects on foliar concentrations of the hormone supplied, but ABA also increased JA-Ile concentrations in all genotypes, and decreased OPDA concentrations in def-1 plants.

Discussion

Although exogenous JA induces stomatal closure (Herde et al., 1997; Hossain et al., 2011; Yin et al., 2016), and water deficit stimulates root (De Ollas et al., 2013; 2015) and shoot (Muñoz-Espinoza et al., 2015) JA and JA-Ile (the bioactive form) accumulation, this study uniquely addresses the physiological significance of JA as a long-distance signal of water deficit. Two complementary approaches were used: allowing reciprocally grafted WT and def-1 tomato plants (which fail to accumulate JA and JA-Ile during water deficit) to dry the soil and supplying detached leaves with xylem-borne JA at the concentrations detected in WT plants grown in drying soil. Although xylem JA concentration of WT plants exposed to water deficit was sufficient to elicit partial stomatal closure when supplied via the xylem to detached WT leaves, attenuating drought-induced root JA export (in WT/def-1 and def-1/WT plants) did not alter stomatal sensitivity to drying soil (Fig. 2C). Only def-1 self-grafts, with the lowest stomatal conductance under well-watered conditions, showed an attenuated stomatal response to drying soil, consistent with their lowest root JA export. Paradoxically, increasing the supply of xylem-borne JA to def-1 shoots (in either detached leaves or def-1/WT plants) increased transpiration, probably by limiting OPDA (a JA precursor) accumulation, which is a more potent antitranspirant than JA (Savchenko et al., 2014). Although spatio-temporal patterns of JA accumulation differed between detached leaf transpiration assays and grafted plants exposed to drying soil, variation in both foliar and xylem sap oxylipin status mediated both constitutive stomatal conductance and stomatal sensitivity to drying soil.

Stomatal responses under well-watered conditions

Well-watered, ungrafted def-1 plants had constitutively lower transpiration rates than either spr2 or WT plants (Table 1). Since all genotypes had similar leaf water potential and xylem and leaf ABA concentration, stomatal morphology
and oxylipin status were measured. Decreased stomatal density of the mutants was compensated by increased stomatal size (Supplementary Fig. S1), making it difficult to attribute variation in transpiration rate to altered stomatal morphology. Alternatively, while spr2 had lower OPDA, JA, and JA-Ile concentrations than WT plants, foliar OPDA concentrations were almost doubled in def-1 plants, supporting the suggestion that OPDA is a more potent antitranspirant than JA/JA-Ile (Savchenko et al., 2014).

Although both mutants failed to accumulate JA as the soil dried (Table 1), def-1 was preferred in reciprocal grafting studies since it had similar JA/JA-Ile concentrations to WT plants under WW conditions. Moreover, def-1 plants had similar vegetative vigour to WT plants, unlike spr2 (Table 1; Supplementary Fig. S4), eliminating the need for a developmental control in soil drying experiments. Although the grafting process itself probably induced transient differences in endogenous JA concentrations, which could inhibit leaf expansion (Moore et al., 2003) of WT scions, it was possible to select reciprocally grafted def-1 and WT plants of similar leaf area to measure transpiration.

Nevertheless, hormonal imbalance in either rootstock or scion decreased transpiration of well-watered plants (Fig. 1) such that grafting WT scions onto def-1 rootstocks decreased their $g_s$ compared with WT self-grafts (Fig. 2), while grafting def-1 scions onto WT rootstocks increased their $g_s$ compared

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**Fig. 9.** ABA (A, B) OPDA (C, D), JA (E, F), and JA-Ile (G, H) concentrations of WT, def-1, and spr2 leaves after 5 h of xylem feeding with artificial xylem sap (open bars), 1000 nM ABA (light grey bars), or 1000 nM JA (dark grey bars) (left panels) or freshly detached leaves before (filled bars) and 5 h after (grey bars) starting the transpiration assay (right panels). Bars are the mean ±SE of four replicates, with different letters denoting significant differences between treatments after Duncan’s HSD, comparing across both panels. The tables summarize the significance ($P$-values) of genotype, treatment, and their interactions after ANOVA of pairwise treatments (artificial xylem sap versus 1000 nM JA and artificial xylem sap versus 1000 nM ABA).
with def-1 self-grafts. These rootstock-mediated changes in gs were correlated with decreased root hydraulic conductivity of def-1 rootstocks (Fig. 5) as previously reported (Sánchez-Romera et al., 2014), yet well-watered plants of all graft combinations maintained a similar leaf water potential (\(\Psi_{\text{leaf}}\)) (Fig. 3A). While homeostatic regulation of \(\Psi_{\text{leaf}}\) by gs may co-ordinate root and shoot conductance (Jones, 1998; Ripullone et al., 2007), it seems unlikely that variation in plant hydraulics caused genotypic differences in transpiration of well-watered plants.

Stomatal responses to drying soil were independent of leaf water status

Genetic variation in stomatal sensitivity to drying soil was assessed by normalizing gs to the maximum (well-watered) value achieved by each graft combination (Fig. 2C). Only def-1 self-grafts (which failed to accumulate JA and its bioactive conjugate JA-Ile as the soil dried) were relatively insensitive to soil drying. Thus WT levels of JA/JA-Ile are required (in either rootstock or scion) to maximize stomatal sensitivity to drying soil, suggesting an antitranspirant effect of JA (Herde et al., 1997). Nevertheless, perturbed leaf hydraulics has been suggested as the most likely mechanism causing stomatal closure (Tardieu, 2016). Although soil drying decreased \(\Psi_{\text{leaf}}\) in all graft combinations, def-1 scions maintained a higher \(\Psi_{\text{leaf}}\) than WT scions (Fig. 3A), independent of root hydraulic conductivity (lower in def-1 rootstocks, irrespective of scion; Fig. 5). This implies that stomatal conductance regulates \(\Psi_{\text{leaf}}\) (rather than vice versa), as in previous tomato experiments where higher gs was associated with decreased \(\Psi_{\text{leaf}}\) in WT plants exposed to various irrigation treatments (Kudoyarova et al., 2007) and in reciprocally grafted, well-watered WT and ABA-deficient plants (Dodd et al., 2009). Taken together, these data imply that phytohormones regulate stomatal closure in response to soil drying (Fig. 10).

Phytohormonal (ABA and JA/JA-Ile) regulation of stomatal response to drying soil

Although an increased foliar and xylem sap ABA concentration has been correlated with stomatal closure in multiple species including tomato (Dodd, 2007; Thompson et al., 2007), def-1 scions required half the foliar ABA concentration to induce maximal stomatal closure (Fig. 7A). Possibly def-1 scions were more sensitive to ABA, but similar stomatal closure of def-1 and WT leaves in response to xylem-borne ABA (Fig. 8; Supplementary Fig. S2) disproves this hypothesis. Stomatal conductance responds more sensitively to xylem sap ABA concentration than foliar ABA accumulation (Geilfus et al., 2015; Tardieu et al., 2015), as much of the latter is compartmentalized in mesophyll cells and is unavailable to the guard cells (Wilkinson et al., 1998). While grafting def-1 scions onto WT rootstocks increased foliar ABA accumulation compared with def-1 self-grafts (Fig. 6A), xylem ABA concentration of def-1 scions was rootstock independent and similar to that of WT scions (Table 2). Taken together, the similar increases in xylem ABA concentration in all graft combinations, yet attenuated stomatal response to drying soil in def-1 self-grafts (Fig. 2C), suggests that other phytohormones were involved in regulating this response.

Restoring normal (WT) stomatal sensitivity to drying soil by grafting def-1 scions onto WT rootstocks was correlated with greater root JA export (Table 2) and partial restoration of foliar JA and JA-Ile status (Fig. 6B, D). Nevertheless, supplemental JA feeding to detached def-1 leaves actually increased their transpiration (Fig. 8), consistent with the higher transpiration of def-1/WT plants than def-1 self-grafts (Fig. 1). Thus stomatal regulation by JA seems dependent on tissue water status, since normal JA levels are required for maximal stomatal conductance in well-watered plants, while drought-induced JA accumulation enhances stomatal closure. Future experiments should establish whether this foliar JA accumulation is a local (induced by decreased \(\Psi_{\text{leaf}}\)) or long-distance (increased root export of JA; Table 2) signal, especially since the xylem JA concentration was more sensitive to drying soil (Fig. 7A), as in previous tomato experiments where higher gs was associated with decreased \(\Psi_{\text{leaf}}\) in WT plants exposed to various irrigation treatments (Kudoyarova et al., 2007) and in reciprocally grafted, well-watered WT and ABA-deficient plants (Dodd et al., 2009). Taken together, these data imply that phytohormones regulate stomatal closure in response to soil drying (Fig. 10).

**Fig. 10.** Model of plant hydraulics and phytohormone impacts on stomatal conductance of grafted plants. Lines ending in arrowheads indicate a positive impact, while lines ending in a bar indicate negative impacts. Root hormone delivery is indicated by dashed lines, while relationships within a biosynthetic pathway are indicated by a yellow arrow.
concentrations detected in WT plants induced partial stomatal closure when fed via the xylem to detached leaves (Fig. 8).

**OPDA affects stomatal responses of plants to drying soil**

Endogenous OPDA concentrations are rarely monitored in plants exposed to water deficit, although they increase even in WT self-grafts (Fig. 6C), and by up to an order of magnitude (Seo et al., 2001; Arbona et al., 2010; Grebner et al., 2013). With soil drying, OPDA accumulation was inversely related to JA accumulation across all graft combinations (cf. Fig. 6B, C), with maximal OPDA levels in def-1 self-grafts. Grafting def-1 scions onto a WT rootstock increased root JA export (Table 2) and decreased foliar OPDA concentrations (Fig. 6C), consistent with suggestions that JA treatment or transport from distal tissues decreases OPDA concentration (Scalschi et al., 2015) by negatively regulating jasmonate biosynthesis or metabolism (Kazan and Manners, 2008). Thus root to shoot JA transport may directly affect transpiration of WT plants (Fig. 1), but may also indirectly regulate transpiration of def-1 scions by decreasing foliar OPDA concentrations (Fig. 6C).

To test the hypothesis that xylem-borne JA/JA-Ile mediates transpiration by limiting foliar OPDA accumulation, JA was supplied via the xylem to detached def-1 and WT leaves. Xylem-supplied JA had opposite effects on both transpiration and foliar OPDA concentrations in the two genotypes. Although xylem-borne JA decreased transpiration of WT leaves, it was significantly less effective than ABA supplied at the same concentrations. In contrast, xylem-supplied JA increased transpiration of def-1 leaves (Fig. 8), consistent with the partial rescue of transpiration in def-1 scions grafted to WT roots (Fig. 1). Whereas xylem-borne JA had no effect on OPDA concentrations in WT leaves, in def-1 leaves increased transpiration was associated with decreased OPDA (high antitranspirant effect) concentrations and increased JA and in planta synthetized JA-Ile (low antitranspirant effect) concentrations (Fig. 9). Taken together, this supports a model in which xylem-transported JA significantly influences g, by regulating accumulation of the JA precursor OPDA (Fig. 10).

**Conclusions**

Although spatio-temporal patterns of JA accumulation differed between detached leaf transpiration assays and grafted plants exposed to drying soil, additional xylem-borne JA consistently decreased stomatal conductance of WT shoots (by activating JA signalling through the COI1 receptor) while increasing stomatal conductance of def-1 shoots (by limiting OPDA accumulation which acts via a COI1-independent pathway). Further work is required to establish the relative importance of these two signalling pathways in WT plants and the distal physiological effects of root to shoot jasmonate transport in plants exposed to water deficit.

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