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Parasitism by *Cuscuta pentagona* sequentially induces JA and SA defence pathways in tomato

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ABSTRACT

While plant responses to herbivores and pathogens are well characterized, responses to attack by other plants remain largely unexplored. We measured phytohormones and C_{18} fatty acids in tomato attacked by the parasitic plant *Cuscuta pentagona*, and used transgenic and mutant plants to explore the roles of the defence-related phytohormones salicylic acid (SA) and jasmonic acid (JA). Parasite attachment to 10-day-old tomato plants elicited few biochemical changes, but a second attachment 10 d later elicited a 60-fold increase in JA, a 30-fold increase in SA and a hypersensitive-like response (HLR). Host age also influenced the response: neither *Cuscuta* seedlings nor established vines elicited a HLR in 10-day-old hosts, but both did in 20-day-old hosts. Parasites grew larger on hosts deficient in SA (*NahG*) or insensitive to JA [*jasmonic acid-insensitive1* (*jai1*)], suggesting that both phytohormones mediate effective defences. Moreover, amounts of JA peaked 12 h before SA, indicating that defences may be coordinated via sequential induction of these hormones. Parasitism also induced increases in free linolenic and linoleic acids and abscisic acid. These findings provide the first documentation of plant hormonal signalling induced by a parasitic plant and show that tomato responses to *C. pentagona* display characteristics similar to both herbivore- and pathogen-induced responses.

Key-words: auxin; *Cuscuta*; fatty acids; induced defences; jasmonic acid; parasitic plant; phytohormones; salicylic acid.

INTRODUCTION

Plant defences induced in response to attack by pathogens and herbivorous arthropods are known to involve complex signalling networks regulated by the plant hormones salicylic acid (SA) and jasmonic acid (JA) (see recent reviews by Durrant & Dong 2004; Glazebrook 2005; Schilmiller & Howe 2005; Wasternack *et al.* 2006; Smith, De Moraes & Mescher 2009). In response to pathogens, SA activates and regulates a hypersensitive response (HR) and initiates the synthesis of an array of antimicrobial phytoalexins and pathogenesis-related (PR) proteins, resulting in systemic acquired resistance (SAR; Durrant & Dong 2004; Pieterse *et al.* 2009). The JA pathway plays a major role in herbivore-induced responses, mediating the production of metabolites that reduce insect growth (Chen *et al.* 2005, 2007; Zhu-Salzman, Luthe & Felton 2008) and of plant volatiles that attract natural enemies (Turlings, Tumlinson & Lewis 1990; De Moraes *et al.* 1998; Dicke 2009) and repel foraging herbivores (De Moraes, Mescher & Tumlinson 2001; Kessler & Baldwin 2001). Much less is known about plant defences induced in response to attack by parasitic plants, despite the fact that these parasites include some of the world's most destructive agricultural pests (Parker & Riches 1993; Musselman, Yoder & Westwood 2001) and have significant impacts on the dynamics of the ecosystems in which they occur (Press & Phoenix 2005; Bardgett *et al.* 2006).

The widespread and recognizable genus *Cuscuta* L. (Convolvulaceae) is one of the most ecologically and economically significant groups of parasitic plants (Kuijt 1969; Dawson *et al.* 1994). *Cuscuta* spp. (dodders) are yellow-to-orange vines that lack roots or expanded leaves and require above-ground attachment to other plants to survive and reproduce (Dawson *et al.* 1994). They generally lack obvious chlorophyll but have retained the genes necessary for photosynthesis, possibly to synthesize lipids for allocation to seeds rather than carbohydrate production (McNeal *et al.* 2007a,b). Upon germination, *Cuscuta* seedlings depend on energy reserves stored in the seeds to grow and forage for hosts. We recently showed that foraging by seedlings of *Cuscuta pentagona* is guided by the perception of host plant volatiles (Runyon, Mescher & De Moraes 2006).

Once a host is located, *Cuscuta* vines twine around the host stem or petiole and the development of haustoria – root-like structures that penetrate host tissues and fuse with the host vascular system to withdraw water and nutrients – begins as *Cuscuta* epidermal cells enlarge and secrete glue-like substances containing primarily de-esterified pectins that adhere to the host (Vaughn 2002, 2003, 2006). It was recently shown that *Cuscuta reflexa* attachment induces the host plant tomato to synthesize an arabinogalactan protein which promotes parasite adherence (Albert, Belasteguí-Macadam & Kaldenhoff 2006). Following attachment, haustorial cells elongate and penetrate the host tissue using both enzymes and mechanical pressure (Nagar, Singh & Sanwal 1984), and individual cells of the haustoria elongate into

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searching hyphae which connect with vascular bundles of the host (Birschwilks *et al.* 2006, 2007). Upon successful formation of vascular connections with the host, *Cuscuta* becomes a powerful sink, withdrawing water, sugars, amino acids and other nutrients (Dawson *et al.* 1994; Birschwilks *et al.* 2007).

Documented host plant responses to attack by *Cuscuta* spp. include a hypersensitive-like response (HLR) and phytoalexin production by a non-host tropical liana in response to *C. reflexa* (Bringmann *et al.* 1999) and the expression of a PR gene by *Cuscuta*-infested alfalfa (Borsics & Lados 2002). Best studied among host plant defences against *Cuscuta* spp. are the responses of resistant tomato varieties to *C. reflexa*, in which elongation of hypodermal host cells, a subsequent HLR and accumulation of phenolics and peroxidases at the attachment site create a mechanical barrier that can block haustorial formation (Ihl *et al.* 1988; Sahm *et al.* 1995). Experimental removal of this dead cell layer allowed the formation of functional haustoria and parasite growth (Ihl *et al.* 1988). Recent molecular work has shown that two aquaporin genes (*LeAqp2*, *TRAMP*) and a cell wall-modifying enzyme (LeXTH1) are expressed in tomato during unsuccessful *C. reflexa* attack, but their roles in defence remain uncertain (Werner *et al.* 2001; Albert *et al.* 2004).

Host defences against parasitic plants whose haustoria attach below ground to host roots are also poorly understood. Application of benzothiadiazole-7-carbothioic acid, a functional analog of SA, promoted resistance of several hosts, including tobacco, to *Orobanche* spp. (broomrapes) (Sauerborn *et al.* 2002; Gonsior *et al.* 2004; Perez-de-Luque, Jorrin & Rubiales 2004; Kusumoto *et al.* 2007). But *Orobanche aegyptiaca* parasitism of tobacco did not induce expression of *PR-1a*, a marker of the SA pathway and SAR (Griffitts, Cramer & Westwood 2004). Reported changes in *Arabidopsis thaliana* gene expression in response to *Orobanche ramosa* include several genes regulated by JA, but not SA-dependent genes (Dos Santos *et al.* 2003a,b). However, treatment with JA analogs did not affect resistance of red clover to *Orobanche minor* (Kusumoto *et al.* 2007). Dos Santos *et al.* (2003a) did report that several genes known to be involved in *Arabidopsis* responses to pathogen attack were induced by *O. ramosa*.

The studies described above provide insights into host plant responses to parasitism, but the specific pathways involved in defence against parasitic plants remain unknown. In the current study, we investigated the induced responses of a susceptible tomato variety to attack by *C. pentagona* by tracking changes in JA, SA, abscisic acid (ABA), auxin (indole-3-acetic acid; IAA) and free linoleic and linolenic acids during the first attachment of recently germinated *C. pentagona* seedlings to a 10-day-old tomato plant and a second attachment 10 d later to the leaf petiole of the same plant. As discussed below, this pattern of attachment is fairly typical of those seen under natural conditions (Runyon, personal observation). Furthermore, we have observed that, during this sequence, the first attachment typically does not induce a HLR, while the second elicits a strong HLR. Finally, because JA and SA, in particular, play well-established roles in plant defence against herbivores and pathogens, we further explored the effectiveness of JA- and SA-mediated responses to *C. pentagona* parasitism by measuring parasite performance on various tomato-signalling mutants.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *C. pentagona* collected from an infested tomato field in Yolo County, California were provided by Dr. T. Lanini (University of California, Davis). Seeds were soaked in concentrated sulphuric acid for 1 h using a Gooch crucible, rinsed for 1 min with distilled water and placed in a Petri dish on moist filter paper to germinate. Tomato plants (*Solanum lycopersicum*) 'Halley 3155', including JA-signalling mutants [*jasmonic acid-insensitive1* (*jai1*), *35S::prosys* and wild-type (WT) 'Castlemart'], were grown in an insect-free growth chamber (25 °C, 16 h photoperiod at 250 μmol m^{-2} s^{-1} provided by cool, white fluorescent tubes) in 9 cm tall × 10 cm square plastic pots filled with a peat-based general-purpose potting soil with fertilizer (Osmocote; The Scotts Company, Marysville, OH, USA). SA-deficient *NahG* and WT 'MoneyMaker' tomato plants were grown similarly, except that they received low light intensity (75 μmol m^{-2} s^{-1}) to prevent development of necrotic leaf spots (Brading *et al.* 2000).

C. pentagona attachment and growth on tomato

Our experimental set-up mimics the progression of parasitism observed to occur in nature, in which newly germinated *Cuscuta* seedlings make an initial attachment to the meristem of very young hosts, and the parasite vine growing from this attachment usually makes a second attachment to the same host, often to the petiole (Runyon, personal observation). Newly germinated *C. pentagona* seedlings, approximately 4 cm long, were allowed to attach to 10-day-old tomato seedlings (first true leaves just beginning to expand; Supporting Information Fig. S1a) by leaning the *C. pentagona* seedling against the right side of the tomato meristem (*Cuscuta* vines coil from right to left). Because far-red light promotes tight coiling of *Cuscuta* spp. (Haidar & Orr 1999), two incandescent 75 W bulbs (75A/CL/DL/RP 120V, Orsam Sylvania, Danvers, MA, USA) per 15 pots in 30 cm × 50 cm flats were placed 1 m above the plants and left on for 24 h (exclusive of a 10 PM to 6 AM scotophase). Using this set-up, *C. pentagona* seedlings coiled tightly around the tomato seedlings within 6 h, and haustorial swellings at points of contact with the host were evident within 24 h. Control plants received the same treatment, and plants exposed to incandescent light for this short period showed no noticeable physiological effects. Ten days later, the growing *Cuscuta* vine was allowed to make a second attachment to the petiole of the second expanded true leaf (the youngest expanded

leaf) of the same now 20-day-old tomato host (Supporting Information Fig. S1b). This was done by repeating brief exposure to incandescent light (as above). After 1–2 h of exposure, the apex of the parasite vines would typically coil, and the site of attachment was controlled by carefully placing the coiled vine around the tomato petiole.

Time course of *C. pentagona* attachment

A time course of phytohormone and free fatty acid changes in tomato was conducted for the first attachment of *C. pentagona* seedlings to 10-day-old tomato plants and for the second attachment to the tomato leaf petiole 10 d later (Supporting Information Fig. S1). All time courses were conducted using tomato variety 'Halley 3155'. The entire tomato seedling was sampled by clipping off the shoot near the soil surface for the first attachment time course (approximately 100 mg). For the second attachment, approximately 100 mg of the tomato petiole, incorporating the *C. pentagona* attachment sites, was sampled and the petiole of a tomato with only the first parasite attachment was sampled as an additional control. In both cases, the parasite vine including haustoria were removed from the host and immediately snap-frozen in liquid nitrogen in Fast-Prep tubes (Q-BIOgene, Carlsbad, CA, USA) with 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Akron, OH, USA), weighed and held at –80 °C until processed. The time courses consisted of nine sampling points: (1) time 0; (2) parasite tightly wrapped, 5–6 h after time 0; (3) first signs of haustoria swelling corresponding to the elongation of *Cuscuta* epidermal cells and adherence of parasite to host, ~24 h after time 0; (4–9) 12, 24, 36, 48, 60 and 120 h after first signs of haustoria development. Six replicates were sampled for every time point, each replicate being a unique parasite–host combination: nine time points × two attachment points × three controls (one for each attachment point + petiole of tomato with only first attachment) × six replicates = 270 tomato plants.

Extraction and quantification of phytohormones and fatty acids

We used vapour phase extraction to measure phytohormones and fatty acids, following a method modified from that of Schmelz *et al.* (2003, 2004). Briefly, plant tissue was homogenized using Zirmil beads (Saint-Gobain ZirPro) in a FastPrep shaker (Q-BIOgene; petioles sampled for the second attachment were ground to a fine powder in liquid nitrogen prior to using Zirmil beads), and the phytohormones were partitioned into an organic layer (dichloromethane), transferred to a 4 mL glass vial, and solvent was evaporated under an air stream. Phytohormones were then derivatized from carboxylic acids to methyl esters using trimethylsilyldiazomethane (Sigma-Aldrich, St. Louis, MO, USA). The vial was heated to 200 °C for 2 min to expedite volatilization of analytes which were collected at this time from the headspace using volatile traps containing 30 mg of Super-Q (Alltech, Deerfield, IL, USA) attached to a vacuum (1 L min^{-1}). The phytohormones were eluted from the traps using 150 μL of dichloromethane and analysed by gas chromatography-mass spectrometry with isobutane chemical ionization with select-ion monitoring (settings described by Schmelz *et al.* 2004). Amounts of free JA, SA, ABA, IAA and linoleic and linolenic acids were quantified using these internal standards (100 ng) added prior to homogenization with beads: $^2H_6$-SA, $^2H_6$-ABA, $^2H_5$-IAA (CDN Isotopes, Pointe-Claire, Quebec, Canada), dhJA (dihydrojasmonic acid; derived via alkaline hydrolysis of methyl dhJA, Bedoukian Research Inc., Danbury, CT, USA) and gamma-linolenic acid (Matreya LLC, Pleasant Gap, PA, USA). Gamma-linolenic was used to quantify linolenic and linoleic acids. Metabolites were analysed on a per-gram-fresh-weight basis and were natural log (ln) transformed to meet variance assumptions. Comparisons were made among treatments for each sampling period in the time courses using one-way analysis of variance (ANOVA); individual means were compared with Tukey's honestly significantly different (HSD) means separation test. All statistics were done using SAS (version 8.2; SAS Institute, Cary, NC, USA).

Performance of *C. pentagona* on JA- and SA-signalling mutants

The biomass of *C. pentagona* on JA and SA tomato mutants was measured 10 d after the first attachment of seedlings to 10-day-old tomatoes, and 10 d after the second attachment to the 20-day-old tomato leaf petiole. At these times, the *C. pentagona* vine and the above-ground tomato shoot were separated, dried in an oven at 55 °C for 72 h and weighed. *Cuscuta* biomass is known to be positively correlated with fitness (Koskela, Salonen & Mutikainen 2001). To determine the effectiveness of putative JA defences, we used well-characterized *jai1* tomato mutants which have lost the function of the tomato homolog of *CORONATINE-INSENSITIVE1*, fail to express JA-responsive genes and have severely compromised resistance to herbivores (Li *et al.* 2004). We also used tomatoes transformed to overexpress prosystemin (*35S::prosys*), a positive regulator of the JA pathway, which exhibit increased resistance to herbivores (Chen *et al.* 2005). Seeds for the *35S::prosys* plants were provided by G. Howe (Michigan State University) and were collected from a *35S::prosys/35S::prosys* homozygote that had been backcrossed five times to the WT 'Castlemart' (Howe & Ryan 1999). To assess SA responses, we used transgenic *NahG* plants expressing the enzyme salicylate hydroxylase, which converts SA immediately to inactive catechol, and are deficient in accumulation of SA (Brading *et al.* 2000). Each combination was replicated 10 times, each replicate being a unique parasite–host combination: five tomato lines (two WTs, three mutants) × two treatments (unparasitized, parasitized) × two attachment points × 10 replicates = 200 tomato plants. Comparisons of biomass were done using one-way ANOVA, and individual means were compared with Tukey's HSD means separation test.

Trypan blue staining of *C. pentagona*-induced HLR cell death

'Castlemart' tomato plants were infested with *Cuscuta* seedlings 15 d after planting by allowing them to attach to the petiole of the third leaf. Plants remained in greenhouse under super spectrum lights for an additional 18 d. Trypan blue staining for detection of plant cell death was based on Koch & Slusarenko (1990) with some modifications. Stems with parasite attachment were removed from plant and submerged in 25% lactic acid, 25% phenol, 25% glycerol and 0.1% trypan blue. Samples were subsequently boiled for 1 min, then removed to destain for 2 h in 25% lactic acid, 25% phenol and 25% glycerol solution. Samples were then transferred to 50% glycerol and photographed.

RESULTS

C. pentagona seedlings induce few changes upon attachment to 10-day-old tomato plants

The first attachment by newly germinated *C. pentagona* seedlings to 10-day-old tomato plants did not alter concentrations of SA, total JA, ABA or free linolenic or linoleic acids (Fig. 1). However, although there was no difference in total JA between parasitized and unparasitized plants, separation of the two JA isomers revealed that *C. pentagona* seedlings did induce a small increase in *cis*-JA 36 h ($P = 0.0070$) and 48 h ($P = 0.0474$) after the development of haustoria began (Fig. 2). The only other parasite-induced change in tomato seedlings documented was an increase in amounts of auxin at 48 h (Fig. 1; $P = 0.0032$); similar *Cuscuta*-induced increases in auxin have been reported

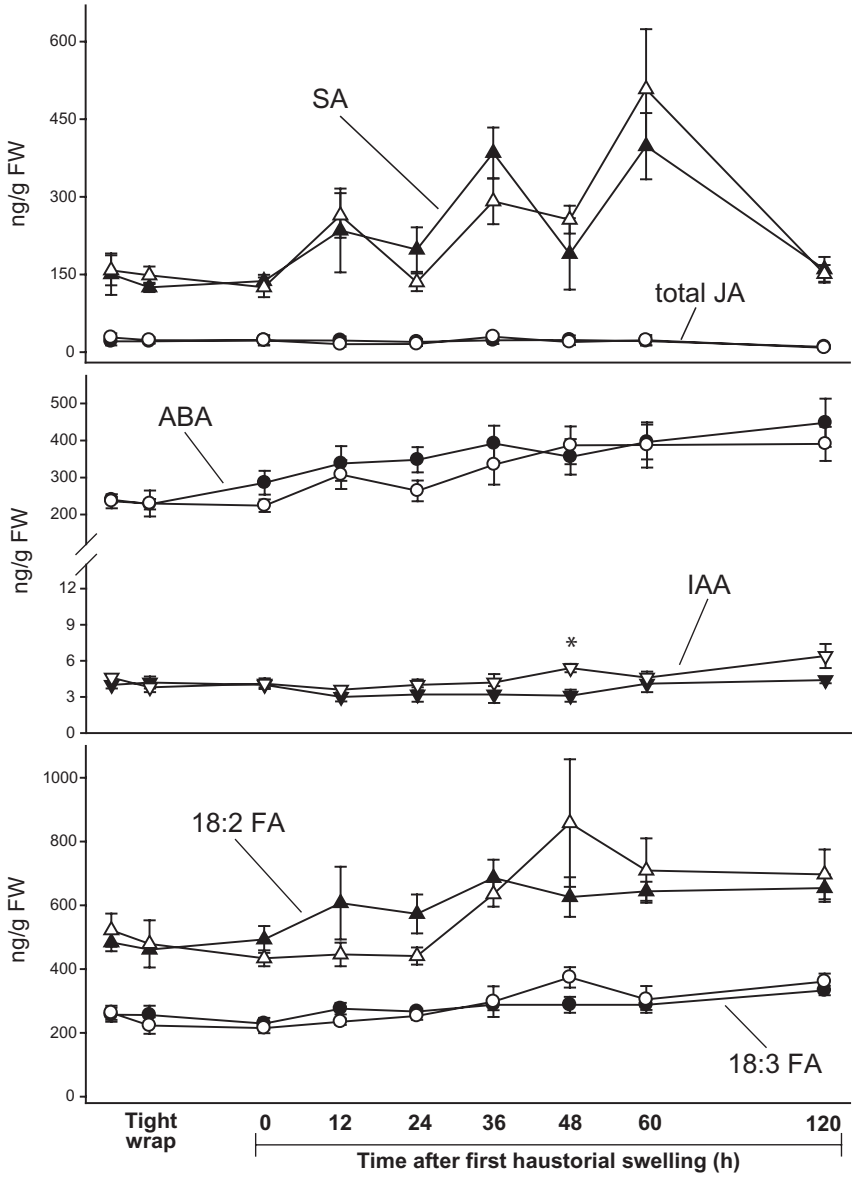

Figure 1. Time course of changes in salicylic acid (SA), total jasmonic acid (JA), abscisic acid (ABA), auxin [indole-3-acetic acid (IAA)], free linoleic acid (18:2 FA) and free linolenic acid (18:3 FA) in 10-day-old tomato plants (mean ± SE, $n = 6$ for each time point; cv. 'Halley 3155') during first attachment by *Cuscuta pentagona* seedlings. Compounds are marked by lines and abbreviations, white symbols represent parasitized plants and dark symbols represent unparasitized controls. Significant difference: $*P < 0.05$. FW, fresh weight.

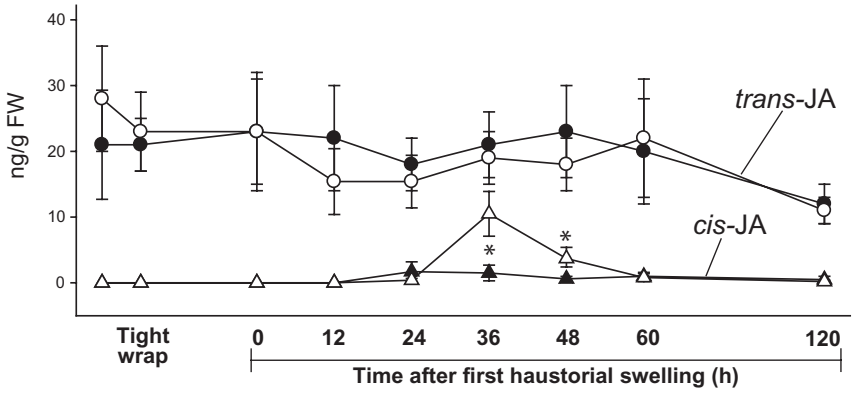

Figure 2. Time course of changes in the *cis*- and *trans*-isomers of jasmonic acid (JA) in 10-day-old tomato plants (mean ± SE, $n = 6$ for each time point; cv. 'Halley 3155') during first attachment by *Cuscuta pentagona* seedlings. Compounds are marked by lines and abbreviations, white symbols represent parasitized plants and dark symbols represent unparasitized controls. Significant differences: $*P < 0.05$. FW, fresh weight.

previously (Werner *et al.* 2001). Parasitism by *C. pentagona* seedlings did not induce a HLR in 10-day-old tomato plants (Fig. 3b). Because we observed that newly germinated parasite seedlings do frequently induce a HLR upon first attachment to 20-day-old tomato plants (Fig. 3a), we conducted a sequence of attachments in which we varied the age of the *C. pentagona* vine and host plant to investigate the conditions influencing the development of a HLR. Neither newly germinated parasite seedlings nor established parasite vines growing from another tomato host elicited a HLR in 10-day-old tomato plants, whereas parasitism of older 20-day-old plants typically induced a strong HLR response (Table 1). We further documented HLR induction by *C. pentagona* using trypan blue, which selectively stains dead plant cells (Fig. 3c,d).

Interestingly, in both time courses, amounts of SA in undamaged tomato plants displayed a diurnal pattern, with SA concentrations generally lower early in the day (time points 0, 24, 48, 120 sampled at approximately 9 AM) and higher late in the day (points 12, 36, 60 sampled at approximately 9 PM) (Figs 1 & 4a). Time-of-day specific gene expression patterns for several phytohormones (SA was not examined) have recently been reported for *Arabidopsis* (Michael *et al.* 2008).

Figure 3. The first attachment by *Cuscuta pentagona* seedlings to the hypocotyls of 20-day-old tomato plants typically elicit a hypersensitive-like response (HLR) (a), but attachment by *C. pentagona* seedlings to the hypocotyls of 10-day-old tomato plants does not result in a HLR (b). Photos were taken 10 d after seedling attachment. Trypan blue staining confirms cell death during HLR induced by *C. pentagona* attachment. (c) Tomato petiole without *Cuscuta* attachment, (d) *Cuscuta* vine 10 d after attachment to petiole of the 3rd leaf (photos by Michelle Peiffer).

Table 1. Elicitation of a HLR by *Cuscuta pentagona* parasitism is influenced by the age of the tomato host (cv. 'Halley 3155')

Cuscuta pentagona vine	Tomato host	Attachment point	Hypersensitive-like response (+/–)	Parasite survival (%)
Newly germinated	10-day-old	Hypocotyl	0/14	100
Established, growing from 20-day-old tomato	10-day-old	Hypocotyl	0/15	100
Newly germinated	20-day-old	Petiole	13/3	50
Newly germinated	20-day-old	Hypocotyl	12/1	46
Established, growing from 20-day-old tomato	20-day-old	Petiole	14/0	100

Newly germinated or established *C. pentagona* vines were allowed to attach to 10- or 20-day-old tomato plants and the presence of a HLR and parasite survival was recorded after 15 d. In all cases, the tomato host was previously unparasitized.
HLR, hypersensitive-like response.

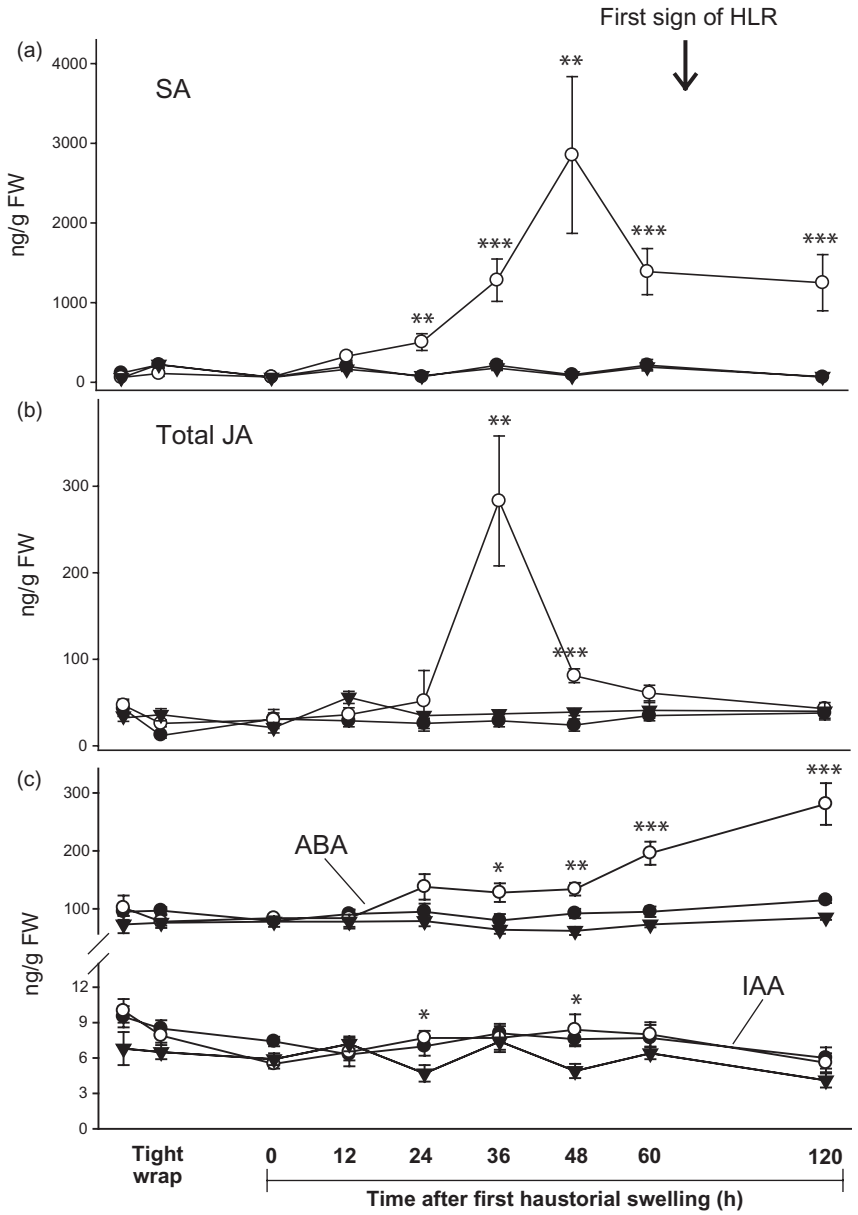

Figure 4. Time course of changes in the phytohormones (a) salicylic acid (SA), (b) total jasmonic acid (JA), and (c) abscisic acid (ABA) and auxin [indole-3-acetic acid (IAA)] in 20-day-old tomato petioles (mean ± SE, $n = 6$ for each time point; cv. 'Halley 3155') during second attachment by *Cuscuta pentagona* seedlings. ABA and IAA are marked by lines and abbreviations. White circles represent parasitized petioles, dark circles represent unparasitized control petioles and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.
HLR, hypersensitive-like response.
FW, fresh weight.

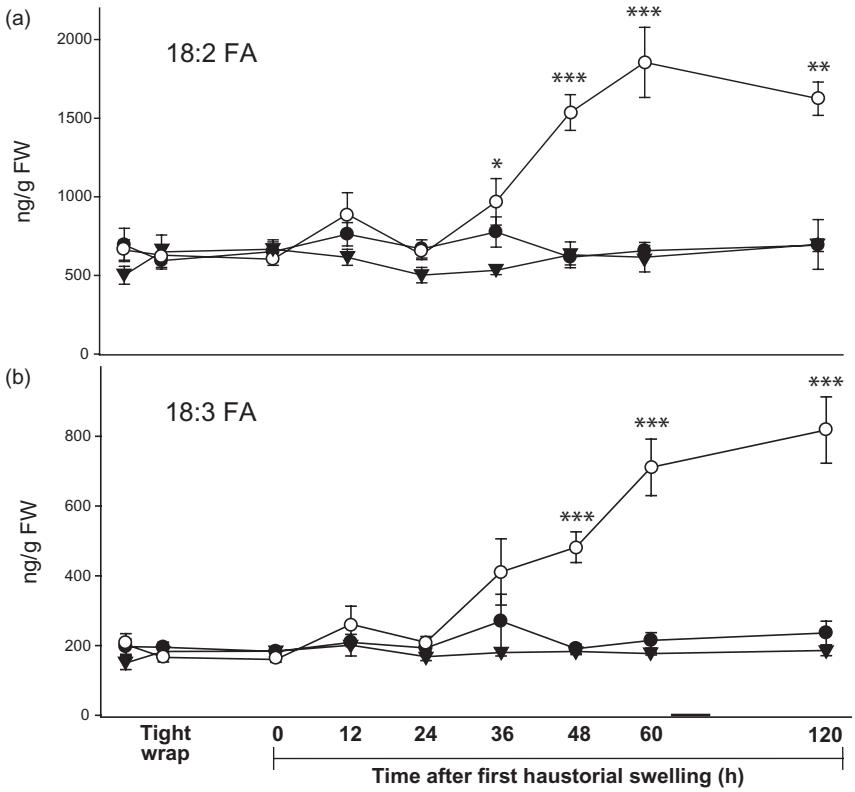

Figure 5. Time course of changes free fatty acids, (a) linoleic (18:2 FA) and (b) linolenic (18:3 FA) in 20-day-old tomato petioles (mean ± SE, $n = 6$ for each time point; cv. 'Halley 3155') during second attachment by *Cuscuta pentagona* seedlings. White circles represent parasitized petioles, dark circles represent unparasitized control petioles and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$. FW, fresh weight.

A second attachment by *C. pentagona* induces SA, JA, ABA and free fatty acids

We next investigated hormonal changes induced in the tomato host when the growing *C. pentagona* vine reattached to the same plant 10 d later. In contrast to the first attachment, the second attachment to a leaf petiole of now 20-day-old tomato plants elicited significant increases in the defensive phytohormones SA and JA, as well as ABA and free fatty acid content (Figs 4 & 5), followed by a strong HLR (Fig. 6, top panel). Amounts of SA began to increase 24 h after the first observed growth of haustoria, reached a maximum at 48 h and remained significantly elevated 5 d after infection began (Fig. 4a). In response to parasitism, total JA increased rapidly between 24 and 36 h after the initiation of haustorial growth, and decreased to control levels by 60 h (Fig. 4b). The change in total JA was primarily the result of an increase in *cis*-JA, which remained significantly elevated 5 d after attachment was initiated (Fig. 7; $P = 0.0142$). The maximum induction of *cis*-JA at 36 h corresponds to the time of maximum induction in 10-day-old plants (Fig. 2). Maximum production of JA by parasitized plants occurred 12 h before that of SA (Fig. 4). In a previous study, we found no *Cuscuta*-induced changes in JA (Runyon, Mescher & De Moraes 2008), but in those experiments, we sampled distal leaf tissue on the parasitized petiole at time points beyond the window of JA production observed here (after 120 h); in the current study, we sampled the petiole at point of parasite attachment. In addition to SA and JA, amounts of ABA were greater in parasitized plants 36 h post-infection and accumulated over time (Fig. 4c). In contrast to the first attachment, amounts of auxin did not increase in the tomato petiole during the second attachment, but petioles of plants with only the first parasite attachment did

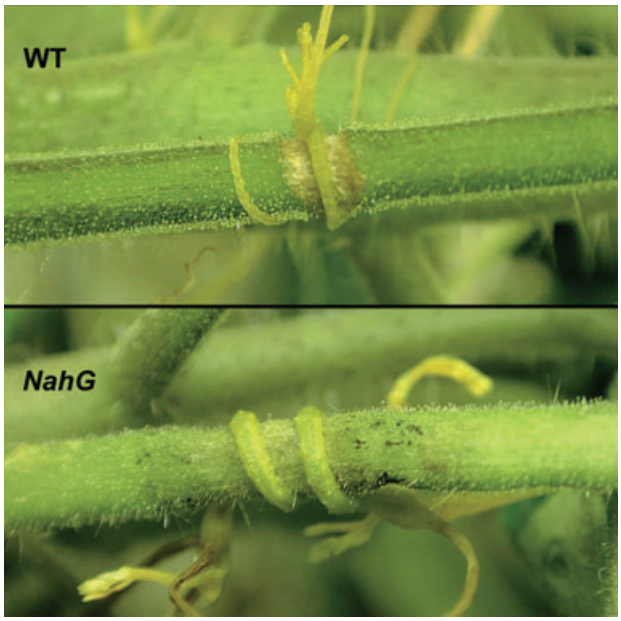

Figure 6. Salicylic acid-deficient tomato plants (*NahG*) do not develop a hypersensitive-like response in response to parasitism by *C. pentagona*. Images are of wild-type (WT) and *NahG* petioles 12 d after second attachment by the *C. pentagona* vine.

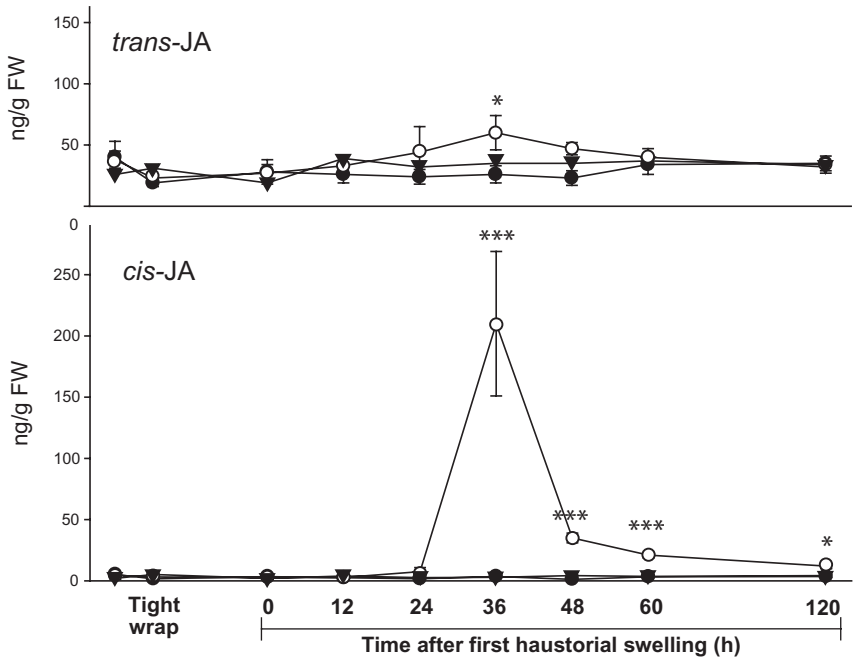

Figure 7. Time course of changes in the *cis*- and *trans*-isomers of jasmonic acid (JA) in 20-day-old tomato petioles (mean ± SE, $n = 6$ for each time point; cv. 'Halley 3155') during second attachment by *Cuscuta pentagona* seedlings. White circles represent parasitized petioles, dark circles represent unparasitized control petioles and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: $*P < 0.05$, $***P < 0.0001$. FW, fresh weight.

contain slightly less auxin at two time points (Fig. 4c). Parasitism by *C. pentagona* also significantly increased free fatty acid content in the host (Fig. 5). In general, amounts of free linoleic and linolenic acids in affected tomato petioles began to increase around 36 h and remained elevated through 120 h (Fig. 5). The first indications of a HLR (indicated by collapsed, but not darkened epidermal cells) appeared about 3 d after haustoria began to enlarge, subsequent to peak increases in JA and SA (Fig. 4). Finally, the petioles of 20-day-old tomato plants with only the first parasite attachment, which were sampled as an additional control, generally did not differ from petioles of unparasitized plants, except for the noted small reduction in auxin at two time points (Fig. 4).

C. pentagona-induced JA and SA appear to mediate effective defences

To assess the impact of JA- and SA-mediated effects on *C. pentagona* performance, we determined the biomass of parasites grown on several tomato mutants with altered JA and SA signalling (1) 10 d after the first attachment to 10-day-old tomato seedlings, and (2) 10 d after the second attachment to the tomato leaf petiole. The biomass of *C. pentagona* grown on WT and SA-deficient *NahG* plants was not different 10 d after the first attachment (Fig. 8a; $P = 0.8478$). There was also no significant difference between the biomass of parasites grown on WT, JA-insensitive or JA-enhanced plants (Fig. 8b; $P = 0.0661$), although the mean parasite biomass on JA-insensitive plants did tend to be greater (Fig. 8b). Although the mean biomass of parasitized tomato plants from all treatments tended to be less than unparasitized plants after only 10 d of parasitism, these differences were also not significant (Fig. 8; $P > 0.05$). Parasite biomass 10 d after the second attachment was significantly greater on both SA-deficient *NahG* and JA-insensitive plants (Fig. 9). Parasites grown on SA-deficient plants were about 40% larger than those grown on the WT (Fig. 9a; mean ± SE biomass: 0.115 ± 0.01 and 0.073 ± 0.01, respectively; $P = 0.0331$). Biomass was also different for parasites grown on the JA-altered and WT plants (Fig. 9b; $P < 0.0001$). Parasitic plants from JA-insensitive plants (*jai1*) were more than twice the size of those grown on WT plants with JA defences intact (Fig. 9b; mean ± SE biomass: 0.310 ± 0.02 and 0.154 ± 0.02, respectively). Mean parasite biomass was lower on plants with the JA pathway constitutively activated (*35S::prosys*) compared to the WT, but the effect was not statistically significant (Fig. 9b; mean ± SE biomass: 0.125 ± 0.02 and 0.154 ± 0.02, respectively; $P > 0.05$). Ten days after the second attachment, *C. pentagona* parasitism had significantly reduced the biomass of all tomato hosts (Fig. 9; $P < 0.001$). Moreover, for the JA-altered plants, biomass of parasitized WT plants was larger than that of *jai1*or *35S::prosys* plants (Fig. 9b). However, it should be noted that *35S::prosys* plants often display a stunted phenotype, perhaps due to the constitutive production of large amounts of JA-inducible defensive proteins (McGurl *et al.* 1994).

Notably, in contrast to the WT, *NahG* plants did not exhibit a HLR after the second attachment by *C. pentagona* (Fig. 6). Also, although a strong HLR was evident in all parasitized WT and JA-overexpressing plants, JA-insensitive plants varied in their production of a HLR to the second attachment: a strong HLR was produced in 40% of the trials, a partial HLR (cell necrosis present only at part of the attachment) in 20% and no HLR in the other 40% ($n = 10$).

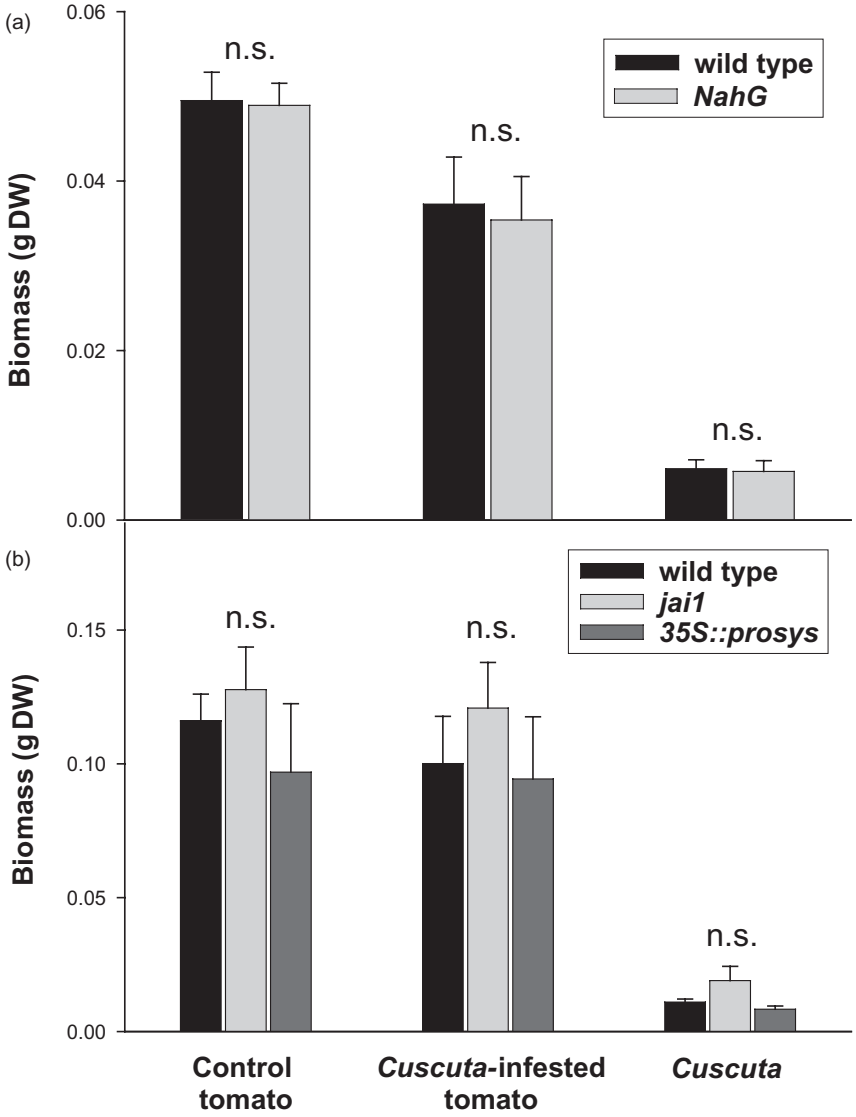

Figure 8. Biomass of *Cuscuta pentagona* and tomato hosts 10 d after first attachment to tomato plants altered in (a) salicylic acid (SA) and (b) jasmonic acid (JA) defence signalling (mean ± SE dry weight, $n = 10$). *NahG* plants are SA-deficient, *jai1* are JA-insensitive and *35S::prosys* constitutively express the JA pathway. DW, dry weight; n.s., no significance between treatments ($P > 0.05$).

DISCUSSION

Despite their ecological and economic significance as plant-feeding organisms, the host defences induced by parasitic plants are not well documented. Here, we used a metabolomic profiling approach involving vapour phase extraction to measure changes in phytohormones occurring within tomato plants during parasitism by *C. pentagona*. Our results indicate that parasite seedlings elicit a relative paucity of host reactions when first attaching to 10-day-old tomato seedlings (Fig. 1), whereas a second attachment by the growing parasite vine 10 d later induces large increases in several plant hormones (Fig. 4) and a strong HLR (Fig. 3a,d). We also assessed the effectiveness of SA- and JA-mediated host changes using transgenic and mutant plants. These methods give the first picture of the composition and timing of hormonal signalling induced in response to a parasitic plant.

First attachment by *C. pentagona* seedlings

The weak changes induced by the first parasite attachment (Figs 1 & 2) contrast sharply with the strong responses elicited during the second attachment (Figs 4, 5 & 7). Our findings that *C. pentagona* seedlings making a first attachment to 20-day-old hosts typically induce a HLR, while established parasite vines do not induce a HLR in 10-day-old tomato plants (Table 1) suggest that the size and age of the host plant is the key factor determining the strength of the response. Reduced survival of newly germinated *C. pentagona* seedlings on older tomato plants appears to corroborate this (Table 1). Field observations showed that seedlings of *Cuscuta gronovii* and *Cuscuta polygonorum* emerge early in the season shortly after their hosts (late April in central Pennsylvania; Runyon, Mescher & De Moraes, unpublished data), which may ensure the presence of young hosts and facilitate successful parasitism. It is

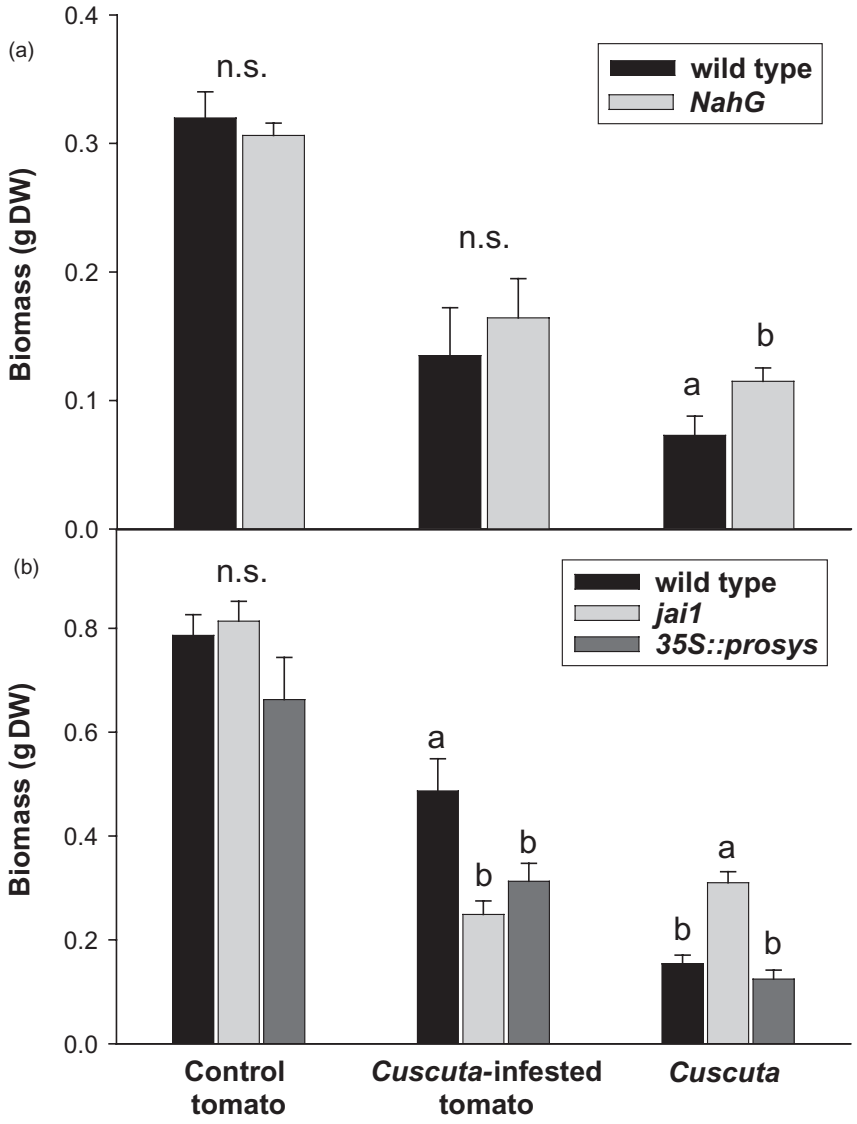

Figure 9. Biomass of *Cuscuta pentagona* and tomato hosts 10 d after second attachment to tomato plants altered in (a) salicylic acid (SA) and (b) jasmonic acid (JA) defence signalling (mean ± SE dry weight, $n = 10$). *NahG* plants are SA-deficient, *jai1* are JA-insensitive and *35S::prosys* constitutively express the JA pathway. Different letters indicate significant differences within treatments ($P < 0.05$). DW, dry weight; n.s., no significance between treatments.

possible that *C. pentagona* seedlings may be better able to avoid or suppress the activation of host responses in younger host plants. Alternatively, plant responses to herbivores can be constrained by ontogeny (Boege & Marquis 2005), and young tomato seedlings may simply be unable to fully respond to parasitism. However, cotyledon-stage tomato plants (similar in size to the 10-day-old plants used in this study) produce large amounts of SA and a HR in response to fungal pathogens (Hammond-Kosack *et al.* 1996).

Alterations of JA or SA in the tomato host had little impact on host or parasite biomass 10 d after the first attachment to 10-day-old tomato plants (Fig. 8). This is likely the result of the early stage of parasitism and the relatively insignificant roles that JA and SA appear to play during this stage of infection (Figs 1 & 2). Although the costs of hosting the parasite 10 d after the first attachment were small, they appeared to be somewhat greater for SA-deficient and WT plants (Fig. 8a) than for JA plants (Fig. 8b), possibly resulting from the low light levels under which SA plants had to be grown to avoid necrotic leaf spots (Brading *et al.* 2000).

Second attachment by *C. pentagona* to tomato petioles

The second attachment by *C. pentagona* induced large increases in the defensive phytohormones SA and JA and a subsequent HLR (Figs 4a,b & 6). SA responses are known to play a role in plant defences against pathogens, including many fungi and bacteria (Durrant & Dong 2004), while JA responses enhance resistance against chewing and sucking herbivores (Walling 2000). We found that *C. pentagona* grew larger on both JA-insensitive and SA-deficient tomato hosts (Fig. 9), indicating that both SA and JA responses may be effective against parasitic plants. Although the defensive compounds that accumulate in response to pathogen-induced SA and herbivore-induced JA have been

characterized (Walling 2000; Durrant & Dong 2004), the mechanisms by which these could operate to reduce *C. pentagona* growth are unknown. However, SA plays a crucial role in the production of a HR (Alvarez 2000), which is an effective defence against *C. reflexa* (Ihl *et al.* 1988). The absence of a HLR in parasitized *NahG* plants (Fig. 6) appears to confirm an essential role for SA in HLR development in this interaction.

Interestingly, a parasite-induced HLR was present in JA WT and *35S::prosys* plants, whereas HLR development was highly variable in *jai1* plants, suggesting that JA also contributes to the HLR. JA has been shown to accumulate in pathogen-induced HR lesions in tobacco (Kenton *et al.* 1999). It is noteworthy that *C. pentagona* induced predominantly *cis*-JA (Fig. 7), which is believed to be the more biologically active of the two naturally occurring forms (Beale & Ward 1998). *cis*-JA is also synthesized in response to insect feeding and application of microbial or insect elicitors (Blechert *et al.* 1995; Engelberth *et al.* 2007). The observed increase in JA provides a plausible explanation for our recent report of increased volatile production by *C. pentagona*-parasitized tomato plants and reduced growth of caterpillars feeding on parasitized tomato leaves (Runyon *et al.* 2008).

The SA- and JA-dependent pathways are known to be antagonistic, and their simultaneous activation can inhibit defence responses. For example, it is well established that SA inhibits the synthesis and action of JA (Peña-Cortes *et al.* 1993; Doares *et al.* 1995; Spoel *et al.* 2003). This cross-talk between the two pathways may allow plants to minimize activation of ineffective defences in favour of operative ones (Rayapuram & Baldwin 2007), or may permit fine-tuning of defences using a combination of both signalling molecules (Reymond & Farmer 1998). Our data show that the production of JA by tomato plants in response to parasitism precedes subsequent accumulation of SA (Fig. 4a,b). The fact that JA and SA appear to independently mediate effective responses against *C. pentagona* (Fig. 9) suggests that tomato plants actively coordinate the synthesis of these hormones to enact a defensive phenotype containing components of both pathways. This may be similar to the sequential use of hormones by tomato against the bacterium *Xanthomonas campestris* pv. *vesicatoria*, in which jasmonate production must precede that of SA to reduce bacterial growth (O'Donnell *et al.* 2003). Although less evidence exists, we also cannot exclude the possibility of synergism between JA and SA, which could result via concentration-dependent interactions (Mur *et al.* 2006).

Amounts of free linolenic and linoleic acids increased in tomato following parasitism by *C. pentagona* (Fig. 5). Free linolenic acid and linoleic acids are known to increase in response to tissue damage by wounding or insect attack, and their oxidation is an early step in the biosynthesis of JA (Conconi *et al.* 1996; Schilmiller & Howe 2005). However, free fatty acid amounts continued to increase well beyond peak JA synthesis at 36 h (Figs 4 & 7), suggesting their induction may have some additional function(s). For example, increases in free linolenic and linoleic acids and their subsequent oxidation could play a role in the development of a HR (Rustérucci *et al.* 1999).

The second attachment by *C. pentagona* also induced increases in ABA (Fig. 4c). ABA typically accumulates in plants under drought stress (Seki *et al.* 2007), and tomato plants may produce ABA in response to removal of water by the parasite. Parasite vines began to grow from the attachment between 36 and 48 h, which corresponds with increases in ABA (Fig. 4c). However, growth of *C. pentagona* from the first attachment at about the same time did not induce ABA in young tomato seedlings (Fig. 1).

In general, the hormone content of *C. pentagona* vines did not differ greatly from that of undamaged tomato plants (Table 2), except that parasite vines growing from the second attachment contained noticeably more ABA (mean ± SE ng g^{-1} ABA: 1929 ± 809 for vines and 340 ± 17 for tomato). A similar distribution of ABA has been reported between *C. reflexa* and a geranium host (De Bock & Fer 1992), and parasitic angiosperms appear generally to contain greater concentrations of ABA than their hosts (Ihl *et al.* 1987; Taylor, Martin & Seel 1996; Jiang, Jeschke & Hartung 2004). It was recently shown that *C. reflexa* can synthesize ABA *de novo* in the absence of a host plant (Qin *et al.* 2008). The function of increased ABA in the parasite is

Table 2. Amounts of SA, JA, ABA, IAA and free linolenic and linoleic acids (ng g^{-1} FW) in *Cuscuta pentagona* seedlings and *C. pentagona* vines growing from the first attachment to 10-day-old tomato seedlings and from the second attachment to 20-day-old tomato petioles (cv. 'Halley 3155'; $n = 6$)

Compound	*C. pentagona* seedlings	*C. pentagona* growing vine 1st attachment	*C. pentagona* growing vine 2nd attachment
Salicylic acid	63 ± 10	206 ± 28	196 ± 30
Total jasmonic acid	7 ± 1	45 ± 9	6 ± 1
trans-JA	6 ± 1	44 ± 8	5 ± 0.7
cis-JA	0.7 ± 0.4	1 ± 1	0.6 ± 0.4
Abscisic acid	54 ± 11	266 ± 32	1929 ± 809
Auxin (IAA)	3 ± 0.2	11 ± 2	14 ± 2
Free linolenic acid	1083 ± 281	418 ± 68	1087 ± 113
Free linoleic acid	1981 ± 458	937 ± 139	778 ± 76

ABA, abscisic acid; FW, fresh weight; IAA indole-3-acetic acid; JA, jasmonic acid; SA, salicylic acid.

unknown, but ABA may increase the flow of water and nutrients (e.g., sucrose) to the parasite (De Bock & Fer 1992; Taylor *et al.* 1996). Alternatively, evidence suggests that ABA could function as a signal in the activation or modification of defences (Bostock 1999; Adie *et al.* 2007; Bodenhausen & Reymond 2007).

Finally, in the compatible *C. pentagona*–tomato interaction, most tomato responses occurred after the parasite began to grow from the host (between 36 and 48 h after visible haustorial swelling). It would be interesting to compare the timing and extent of tomato responses to *C. pentagona* with those to *C. reflexa*, in which tomato plants successfully prevent parasitism by blocking initial haustorial growth using a HLR. Perhaps, like plant interactions with pathogens, the speed of host perception and responses to parasitic plants can determine resistance or susceptibility.

In summary, we conclude that as with herbivore and pathogen attack, plants are able to perceive invasion by parasitic plant haustoria and respond by activating induced defence pathways. Seedlings of *C. pentagona* elicited relatively few changes in the host upon first attachment to young tomato seedlings, possibly because of ontogenetic constraints in host defence or because the parasite is better able to manipulate young hosts. Older tomato plants responded to a second attachment by activating the JA- and SA-signalling pathways, both of which appear to mediate defences that effectively reduce parasite growth. Moreover, our results suggest that by varying the timing of JA and SA synthesis, parasitized plants may achieve a defensive response containing elements of both pathways. Parasitism also induced increases in ABA and free fatty acids, but the roles of these compounds in defence remain uncertain.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic of experimental set-up: (a) first attachment of *Cuscuta pentagona* seedlings to 10-day-old tomato plant, and (b) second attachment by growing parasite from first attachment to youngest petiole of now 20-day-old tomato plant. For time courses, most of the above-ground tomato seedling was sampled for first attachment (a), and the parasitized petiole was sampled for the second attachment (b; indicated by a black oval). Tomato variety 'Halley 3155' was used for all time courses. For growth assays investigating performance of *C. pentagona* on tomato-signalling mutants, host and parasite biomass were determined 10 d after first attachment (a) and 10 d after second attachment (b).

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