



# Stem parasitic plant *Cuscuta australis* (dodder) transfers herbivory-induced signals among plants

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*Cuscuta* spp. (i.e., dodders) are stem parasites that naturally graft to their host plants to extract water and nutrients; multiple adjacent hosts are often parasitized by one or more *Cuscuta* plants simultaneously, forming connected plant clusters. Metabolites, proteins, and mRNAs are known to be transferred from hosts to *Cuscuta*, and *Cuscuta* bridges even facilitate host-to-host virus movement. Whether *Cuscuta* bridges transmit ecologically meaningful signals remains unknown. Here we show that, when host plants are connected by *Cuscuta* bridges, systemic herbivory signals are transmitted from attacked plants to unattacked plants, as revealed by the large transcriptomic changes in the attacked local leaves, undamaged systemic leaves of the attacked plants, and leaves of unattacked but connected hosts. The interplant signaling is largely dependent on the jasmonic acid pathway of the damaged local plants, and can be found among conspecific or hetero-specific hosts of different families. Importantly, herbivore attack of one host plant elevates defensive metabolites in the other systemic *Cuscuta* bridge-connected hosts, resulting in enhanced resistance against insects even in several consecutively *Cuscuta*-connected host plants over long distances (> 100 cm). By facilitating plant-to-plant signaling, *Cuscuta* provides an information-based means of countering the resource-based fitness costs to their hosts.

*Cuscuta* | parasitic plant | systemic defense | herbivore | jasmonic acid

It has long been recognized that plants react to insect feeding in a highly sophisticated and well-organized manner. When a leaf is attacked, it responds to the wounding, and, in some cases, certain insect-derived elicitors that are introduced into wounds during attack, with a series of rapidly activated signaling events, including MAPK activation, Ca<sup>2+</sup> influxes, and jasmonic acid (JA) biosynthesis, which eventually lead to the accumulations of defense metabolites that enhance defense against further attack (1, 2).

As an integrated system, different parts of a plant, such as individual leaves, shoots, and roots, constantly communicate to coordinate growth and development, as well as respond to environmental stresses, including herbivory. Insect herbivory not only activates defenses at the site of feeding, but also induces unknown mobile signals that travel through vasculatures to other parts of the damaged (local) leaf and even to undamaged (systemic) leaves and roots, triggering systemic defense responses (1, 3–5). Systemic defense was first discovered in the tomato (*Solanum lycopersicum*): after leaf wounding, systemic leaves were found to increase the production of an important defensive metabolite, proteinase inhibitor (PI)-I (6). Since then, much research has revealed that, after wounding or insect feeding, systemic signaling plays an important role in regulating defense-related responses in systemic tissues. However, the nature of the mobile systemic signals is not fully understood. Reciprocal grafting experiments using WT and mutant tomato plants deficient in JA production and perception indicate that induction of *PI-II* in systemic leaves requires the capability of JA biosynthesis in local leaves and perception in systemic leaves, demonstrating the involvement of JA in wounding-induced systemic signaling (7). Wounding *Arabidopsis* leaves also increases the contents of JA and JA-isoleucine con-

jugate in systemic leaves within minutes (8). Moreover, reactive oxygen species (ROS) were found to be produced in local and systemic leaves of *Arabidopsis* and appeared to play a role in the propagation of systemic signals (9), and several studies have pointed to the involvement of electric signals in wounding- and insect feeding-induced systemic signaling (10–12).

*Cuscuta* spp. (i.e., dodders) are stem holoparasites with wide host ranges. *Cuscuta* seedlings are thread-like (without roots and cotyledons), and, after contacting hosts, they form initial haustoria, organs that penetrate into hosts, transporting various substances, including water and nutrients, from hosts to the parasites. Subsequently, seedlings rapidly develop into tendrils, which coil around hosts, continuously forming new haustoria and branching vines. During a vigorous growth stage, *Cuscuta* vines often extend from the initial hosts and parasitize neighboring plants, forming clusters of plants that are connected by one or more *Cuscuta* parasites.

Recent studies have shown that certain host metabolites, proteins, and mRNAs can be detected in *Cuscuta* (13–16), and even viruses can be transferred from one host to another through *Cuscuta* bridge connections (17). For example, glucosinolates (GSs) in *Arabidopsis* can be translocated to *Cuscuta gronovii* and protect this parasite against pea aphids (*Acyrtosiphon pisum*)

## Significance

*Cuscuta* spp. (i.e., dodders) are plant parasites that connect to the vasculature of their host plants to extract water, nutrients, and even macromolecules. Knowledge of ecologically meaningful communications between host plants and *Cuscuta*, or between *Cuscuta* bridge-connected hosts, has remained obscure until now. Here we show that herbivore attack on one of the *Cuscuta* bridge-connected plants induces gene expression and increases the activity of trypsin proteinase inhibitors, and thus elevates the resistance to insects in other undamaged but *Cuscuta*-connected plants. This *Cuscuta*-mediated interplant signaling is rapid, conserved, far-reaching, and partly requires the plant hormone jasmonic acid. Although *Cuscuta* parasites can negatively influence their host plants, under certain circumstances, they may also provide ecologically relevant information-based benefits.

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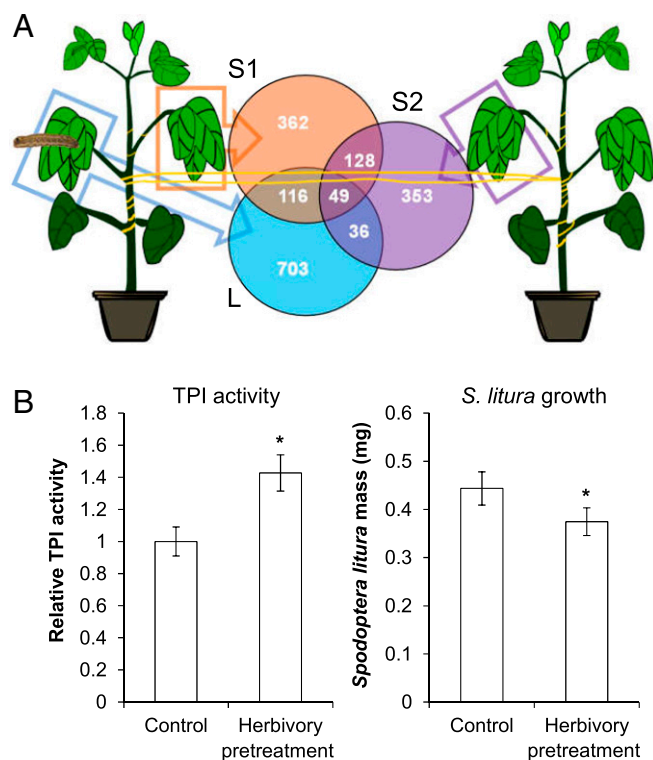
(16). Similarly, a hemiparasite, *Castilleja indivisa* (Scrophulariaceae), also transports alkaloids (mainly lupanine) from lupine (*Lupinus texensis*), and these hemiparasites exhibited decreased herbivore damage, increased visitation by pollinators, and lifetime seed production (18, 19). However, whether ecologically meaningful signals are communicated among hosts and *Cuscuta* and even among hosts in *Cuscuta*-connected plant clusters is unknown. Here we demonstrate that, in these clusters, *Cuscuta* mediates herbivory-induced interplant signaling that activates defense responses and primes the undamaged host plants in *Cuscuta* bridge-connected hosts against subsequent insect attack.

## Results

**Insect Feeding Induces Transcriptomic Reconfigurations in All Members of *Cuscuta* Bridge-Connected Clusters and Elicits Resistance in Connected but Unattacked Host Plants.** Vasculature conveys systemic signals within plants, and, given that *Cuscuta* parasites fuse to a host's vasculature system, we tested whether insect feeding on one host plant could induce defense responses in the other unattacked/systemic but *Cuscuta*-connected hosts. Soybean (*Glycine max*) plants were infested with *Cuscuta australis* vines, and, during the vigorous growth stage, pairs of soybean plants were created by placing two *C. australis*-infected soybean hosts next to each other and allowing *C. australis* vines to parasitize and connect the two hosts. When the soybean plant clusters were established, soybean leaves (local leaves; named L leaves) were infested with *Spodoptera litura* larvae or left untreated (controls). After 24 h of feeding, the L leaves, the systemic leaves of the caterpillar-attacked plants (S1 leaves), the leaves of the connected but undamaged systemic plant (S2 leaves), and the vines of *C. australis* were harvested for RNA-sequencing (RNA-seq) analysis (Fig. 1A).

Compared with the control group, 904 genes were up- or down-regulated at least onefold (820 and 84, respectively) in the L leaves after *S. litura* infestation, and the S1 leaves had 655 genes with altered transcript levels (519 up- and 136 down-regulated), consistent with the activity of systemic signals moving from the local to systemic leaves within the attacked plants. Importantly, 566 genes (283 up- and 283 down-regulated) were regulated in S2 leaves (Fig. 1A; genes are listed in Dataset S1), suggesting that *C. australis* bridge connections allowed herbivory-induced systemic signals to reach the connected second hosts. Most genes in the L and S1 leaves were induced (approximately 90% and 80%, respectively), whereas only 50% were induced in the S2 leaves (Dataset S1). The regulated genes had little overlap: (i) 703, 362, and 353 genes responded specifically in L, S1, and S2 leaves; and (ii) only 49 genes were commonly regulated, among which seven showed different directions of expression level changes between L, S1, or S2 leaves (Fig. 1A and Fig. S1). Importantly, two genes, *Glyma09g28310* and *Glyma16g33710*, which encode trypsin protease inhibitors (TPIs; important antiinsect proteins), were up-regulated in all L, S1, and S2 leaves, suggesting that, in response to herbivory, all *Cuscuta*-connected soybean plants in a cluster may have elevated defense levels.

To elucidate the biological processes that were involved in the response to *S. litura* feeding on L leaves, overrepresentation analyses on the differentially expressed genes (DEGs) were performed using MetGenMap (20). In the L leaves, three pathways were strongly ( $P < 0.001$ ) enriched: "jasmonic acid biosynthesis," "divinyl ether biosynthesis II," and "13-LOX and 13-HPL pathway" (Table S1). The DEGs in S1 leaves were strongly enriched in "cellulose biosynthesis," "jasmonic acid biosynthesis," and three "asparagine biosynthesis" pathways (Table S1). In the S2 leaves, "glycerol degradation I" and "cellulose biosynthesis" were the most significantly ( $P < 0.001$ ) enriched pathways (Table S1). It is noteworthy that the "monoterpene biosynthesis" pathway was regulated ( $P < 0.05$ ) in all leaves, and monoterpenes may function as an indirect defense by attracting the predators of herbivorous insects (21). Furthermore, almost all significantly enriched pathways



**Fig. 1.** *S. litura* feeding induces transcriptional changes and increases defense and resistance levels in leaves of undamaged but *Cuscuta*-connected soybean plants. (A) Venn diagrams depicting the numbers of differentially regulated genes in L, S1, and S2 leaves (detailed descriptions of these genes are provided in Dataset S1). After 24 h of *S. litura* feeding on the L leaves, the L leaves, S1 leaves, S2 leaves, and the vines of *C. australis* that connected both host plants were harvested for RNA-seq analyses ( $n = 3$ ). (B) The TPI activity and resistance levels of the systemic host plants. L leaves were infested with *S. litura* for 48 h (herbivory pretreatment) or left untreated (control), and S2 leaves from both groups were then harvested for TPI activity quantification ( $n = 6$ ; Left), or were infested with *S. litura* larvae ( $n = 35$ ), with insect masses determined after another 72 h (Right). Values in the control group are normalized to 1; values are means  $\pm$  SE, and asterisks indicate significant differences between plants of the control group and herbivory pretreatment group ( $*P < 0.05$ ,  $t$  test).

in S2 leaves could be found in S1 leaves, except "glycerol degradation I," suggesting that S1 and S2 leaves had similar defense-related responses.

The RNA-seq analysis of *C. australis* vines revealed 140 DEGs (79 and 61 up- and down-regulated, respectively) after *S. litura* feeding on L leaves (Dataset S2), including genes encoding proteins with leucine-rich repeats, cytochrome P450s,  $Ca^{2+}$ -binding proteins, and proteins from the superfamily of protein kinases. The most significantly altered pathways were all related to primary metabolism (Table S1).

These data indicate that *Cuscuta* bridge connections between different hosts enable a new type of interplant systemic signaling, inducing transcriptome rearrangements in the systemic undamaged hosts. To examine if these large transcriptomic changes in the systemic plants are associated with enhanced herbivore resistance, soybean-*C. australis*-soybean plant clusters were established, L leaves were treated with *S. litura* feeding or left untreated (pretreated group and control group, respectively), and the S2 leaves of the systemic soybean plants were examined for the activity of defense metabolite TPI and insect resistance. After 48 h of *S. litura* feeding on L leaves, the TPI activity in the S2 leaves was ~40% greater than in the control group (Fig. 1B), and these S2 leaves with enhanced defense consistently exhibited elevated

resistance to insects, as indicated by the fact that, 3 d after infesting S2 leaves, the masses of *S. litura* neonates were ~16% lower than those on the S2 leaves of the control group (Fig. 1B). Given that hundreds of DEGs were detected in S2 leaves, it is likely that other induced defenses, in addition to TPIs, also contributed to the elevated resistance to *S. litura*.

Plants release volatile organic compounds in response to wounding and herbivore attack, some of which may elicit resistance-related reactions in neighboring plants (21). To rule out the possibility that airborne signals from the L leaves induced the aforementioned responses in the S2 leaves, pairs of *C. australis*-infected soybean plants were created, but, this time, *C. australis* parasites were not allowed to bridge connect the host pairs, and, in each soybean plant pair, one was left untreated or fed on by *S. litura* for 24 h. RNA-seq revealed that there were only 26 DEGs in the S2 leaves (Dataset S3), and one of these, a nakin-2-like gene (*Glyma04g35030*), was significantly changed regardless of whether there were *Cuscuta* bridges, but in opposite directions (Datasets S1 and S3). Consistently, when soybean pairs were not connected by *C. australis*, neither TPI activity nor increased resistance could be detected in the untreated host plants (Fig. S2). Moreover, when we examined whether there are airborne signals between two soybean plants, which were not parasitized at all (Dataset S4), only 35 DEGs were found, and none of these genes were in common with the DEGs in the other two setups (Datasets S1 and S3).

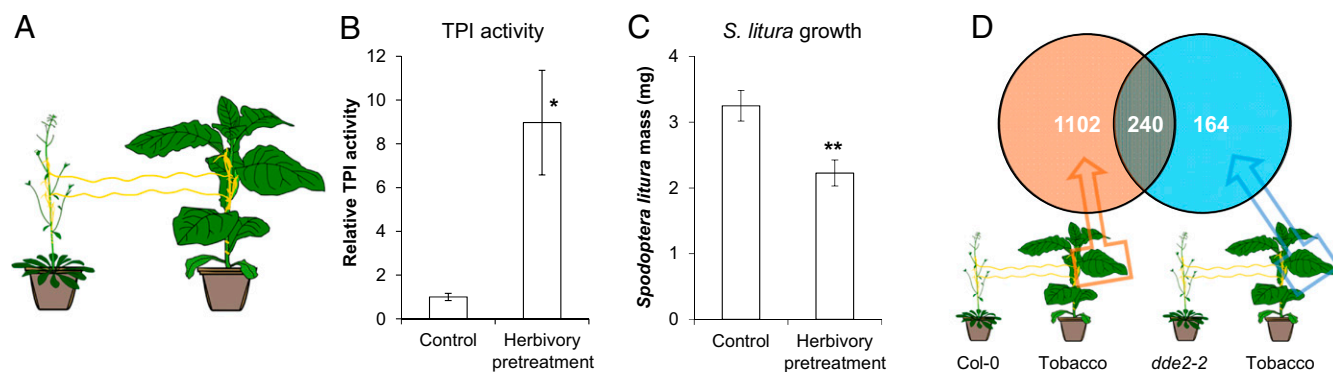
The DEGs identified in these experiments likely resulted from random errors (22), and parasitization did not appear to oversensitize soybean to the airborne signals. Thus, a role for airborne signals in mediating the between-plant signaling could be ruled out.

**Cuscuta-Mediated Interplant Signaling Is Well Conserved and Involves JA Pathway.** As the host plants in the *Cuscuta*-connected plant clusters can be different species, we evaluated whether insect feeding increases the defense levels of systemic plants in a heterospecific hosts system. Plant clusters, each of which consisted of an *Arabidopsis* (Brassicaceae)–*C. australis*–tobacco (Solanaceae) combination (Fig. 2A), were established. *Arabidopsis* plants were treated with *S. litura* feeding for 48 h (pretreated group) or untreated (control group). Compared with those in the control group, the TPI activity levels in the systemic tobacco plants of the pretreated group increased eightfold (Fig. 2B). The tobacco plants in both groups were infested with *S. litura* for another 72 h to evaluate their resistance levels, and it was found that, compared with those in the control group, the average mass of

*S. litura* in the pretreated group was 32% smaller (Fig. 2C). Without *C. australis* bridge connections, after 48 h of *S. litura* feeding on *Arabidopsis*, the neighboring tobacco plants showed no signs of elevated TPI activity and, consistently, these tobacco plants did not exhibit increased resistance to *S. litura* (Fig. S3). Similarly, in soybean (Fabaceae)–*C. australis*–wild tomato (*Solanum pennellii*; Solanaceae) plant clusters, 8 h after soybean leaves were treated with *S. litura* feeding, the transcript levels of *PI-II* (encoding defensive PIs) in the wild tomato were elevated more than twofold (Fig. S4A). Again, treatment of soybean did not affect *PI-II* transcript abundance in unconnected wild tomato plants, allowing airborne signals to be ruled out (Fig. S4B).

Several studies have indicated the involvement of JA in systemic defense responses (7, 8, 23). To investigate the role of JA in *Cuscuta*-mediated systemic signaling between plants, WT (Col-0) and *dde2-2 Arabidopsis* (24), which harbors a mutation in the JA biosynthesis gene *AOS* (*allene oxide synthase*), were connected pairwise to tobacco plants. *Arabidopsis* plants were untreated or wounded, and, after 8 h, the leaves of the tobacco were harvested for RNA-seq analysis (Fig. 2D). Wounding was chosen instead of insect feeding because *dde2-2* mutant is completely impaired in JA biosynthesis; therefore, insects likely cause much greater amounts of damage to *dde2-2* plants than WT plants, thereby complicating the analysis.

Compared with the untreated control group, 1,342 DEGs (981 up- and 361 down-regulated, respectively) were identified in the tobacco of the wound-treatment group (Fig. 2D and Dataset S5), and the most strongly enriched pathways included “13-LOX and 13-HPL” and “jasmonic acid biosynthesis” (Table S2). In contrast, wounding *dde2-2* resulted in only 404 DEGs (191 up- and 213 down-regulated) in the tobacco (Fig. 2D and Dataset S5), and “nitrate reduction II (assimilatory),” “ammonia assimilation cycle II,” and “glutamine biosynthesis I” were among the enriched pathways (Table S2). Notably, three genes coding trypsin protease inhibitors, *SS15144g00001*, *SS72472g00002*, and *SS2003g01003*, were up-regulated (18, 2, and infinite times, respectively; the expression level of *SS2003g01003* was not detectable in controls but relatively high after induction, resulting in an infinite ratio) in the tobacco connected with Col-0, whereas only *SS15144g00001* was induced threefold when the tobacco plants were connected with *dde2-2* (Dataset S5). Moreover, Venn-diagram analyses indicated that 240 tobacco genes were commonly regulated in the tobacco plants in both Col-0–*Cuscuta*–tobacco and *dde2-2*–*Cuscuta*–tobacco plant clusters (Fig. 2D). Among these, 115 genes showed very similar patterns of regulation (the differences between



**Fig. 2.** *Cuscuta*-mediated interplant signaling occurs between heterospecific hosts and involves the JA pathway. (A) Schematic of an *Arabidopsis*–*C. australis*–tobacco plant cluster. *Arabidopsis* leaves were infested with *S. litura* for 48 h (herbivory pretreatment) or untreated (control). Tobacco leaves were harvested for determination of relative TPI activity ( $n = 6$ ; values in the control group are normalized to 1) (B), or infested with *S. litura* ( $n = 25$ ) and, after 72 h of feeding, the masses of these insects were determined (C). Values are means  $\pm$  SE, and asterisks indicate significant differences between controls and the herbivory pretreatment group (\* $P < 0.05$  and \*\* $P < 0.01$ ,  $t$  test). (D) Venn diagram depicting the specifically and commonly regulated genes in tobacco plants connected with *Arabidopsis* WT (Col-0) or the JA-deficient *dde2-2* plants. Col-0 and *dde2-2* were untreated or wounded with a pair of forceps, and tobacco samples were harvested after 8 h to analyze the transcriptional responses by RNA-seq (details are provided in Dataset S5).

the levels of wound-induced changes in these two plant clusters were not more than 50% (Dataset S5). Likely, transcriptional regulation of these 115 genes does not require the JA pathway in the local plants, whereas the expression of the other 125 genes is probably partly dependent on JA. Furthermore, 164 genes were uniquely regulated in the tobacco of the *dde2-2-Cuscuta*-tobacco plant clusters (Fig. 2D), and we speculate that these genes were regulated by systemic signal(s) that is/are normally suppressed by the JA pathway. In line with these RNA-seq data, *S. litura* feeding on the *dde2-2* mutants could no longer prime the connected tobacco plants against subsequent insect attack (Fig. S5).

Based on these results, we concluded that *Cuscuta* bridge connections transmit conserved insect herbivory-induced systemic signals among different conspecific or heterospecific hosts, and, importantly, this interplant signaling plays an important role in priming all connected hosts in clusters against insect attack. Furthermore, the JA pathway in the local wounded plants is crucial in initiating and/or maintaining the systemic signals, and it is possible that there is more than one mobile signal that could travel through *Cuscuta* bridges between plants.

**Insect-Derived Elicitors Amplify Wound-Induced Defenses in *Cuscuta*-Connected Systemic Plants.** Many solanaceous plants, such as tobacco (*Nicotiana tabacum*), are able to perceive insect oral secretions (OS) and activate stronger defense responses than after mechanical wounding does alone in local and systemic tissues (1). To evaluate whether the perception of insect-derived elicitors induces higher levels of defenses in *Cuscuta* bridge-connected systemic plants than wounding does alone, three different groups of *C. australis*-connected tobacco pairs (named Tobacco 1 and 2) were established: control untreated (n.t.) and pairs in which Tobacco 1 was wounded and treated with water (W+W) or treated with *S. litura* OS (W+OS; Fig. 3). The leaves of the systemic Tobacco 2 plants were harvested after 8 h; *TPI* transcript levels increased in the W+W-treated group, and these levels were amplified in the W+OS-treated group (Fig. 3). Again, no evidence of airborne signals was found in tobacco pairs arranged similarly, but without *C. australis* bridge connections (Fig. S6).

From these results, we infer that the interplant systemic signals can be amplified after perception of the elicitors in insect OS and pass through *Cuscuta* to activate herbivory-specific defense responses in systemic host plants.

***C. australis*-Mediated Interplant Signaling Is Rapid and Can Be Transmitted over Long Distances.** The early systemic responses to wounding have been well studied in *Arabidopsis* plants, and

several systemic marker genes that rapidly respond to wounding/herbivory are known (9). To determine how rapidly this form of interplant signaling can occur between *C. australis* bridge-connected hosts, two *Arabidopsis* plants (*Arabidopsis* 1 and 2) were infested with *C. australis* to form *Arabidopsis*-*C. australis*-*Arabidopsis* plant clusters (Fig. 4A). *Arabidopsis* 1 was wounded to mimic insect feeding (insect feeding behavior is difficult to control; thus, simulated herbivory was used to precisely synchronize the elicitation). Transcriptional responses in *Arabidopsis* 2 were detected as early as 30 min after the wounding of *Arabidopsis* 1, when the transcript abundances of three wound-inducible systemic marker genes (9), *ZAT12*, *WRKY40*, and *WRKY53*, increased several fold in the leaves of *Arabidopsis* 2 (Fig. 4A). Similarly, W+OS treatment of tobacco in heterospecific tobacco-*C. australis*-*Arabidopsis* plant clusters increased the transcript levels of *WRKY40* in the neighboring *Arabidopsis* approximately 10-fold at 45 min (Fig. S7). Thus, the systemic signal travels in a relatively high speed, approximately 1 cm/min, inferred from the distances between the hosts.

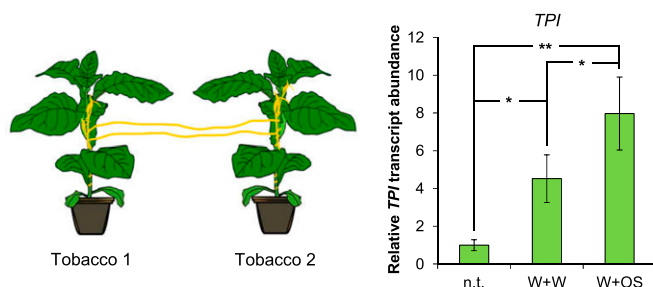
In nature, one or multiple *Cuscuta* plants can simultaneously parasitize multiple hosts in neighborhoods, forming interconnected plant clusters (25). To determine whether *Cuscuta*-mediated interplant signaling can occur over multiple plants connected by different *Cuscuta* parasites, six *Arabidopsis* plants (A1–A6; ~100-cm total distance) were pairwise connected in a row by five individual *C. australis* (Fig. 4B). After the first plant (A1) was wounded, the transcript levels of *ZAT12*, *WRKY40*, and *WRKY53* in A2–A6 were determined. At 90 min, these genes' transcript levels in the A4 plants (Fig. 4B) were similar to those in *Arabidopsis* 2 plants of the connected pairs of plants at the 45-min time point (Fig. 4A), and the transcript levels in A2 were already decreasing at 90 min, suggesting that the response was propagated as a wave through the connected plants. As A6 started to show detectable responses, we conclude that the *Cuscuta*-mediated interplant signaling can occur along multiple hosts reaching at least ~100 cm. We did not find any evidence that airborne signals could induce gene expression in *Arabidopsis*: without *Cuscuta* bridge connections, wounding an *Arabidopsis* did not influence transcript levels of these marker genes in adjacent *Arabidopsis* plants (Fig. S8).

We next examined whether the interplant signaling can activate defenses in the distal plants. As establishing *Cuscuta* bridge connections between plants is a lengthy process, *Arabidopsis* plants had started to flower by the time they were well connected by *Cuscuta*, and, at this stage, the levels of systemic GSs were no longer inducible by *S. litura* feeding (Fig. S9). Therefore, we replaced the A6 *Arabidopsis* with a tobacco plant, the *TPI* activity of which can still be induced after establishment of plant clusters. The *Arabidopsis* A1 was infested with *S. litura*, and, after 48 h, tobacco leaves were harvested for determination of *TPI* activity or infested with *S. litura* for another 72 h. The *TPI* activity in these tobacco plants was more than twofold greater than in the nontreated control group (Fig. 4C), and these plants consistently exhibited elevated resistance, as the masses of *S. litura* neonates were 20% lower than those infested on the controls, which were not pretreated with 48 h of *S. litura* feeding on A1 (Fig. 4C).

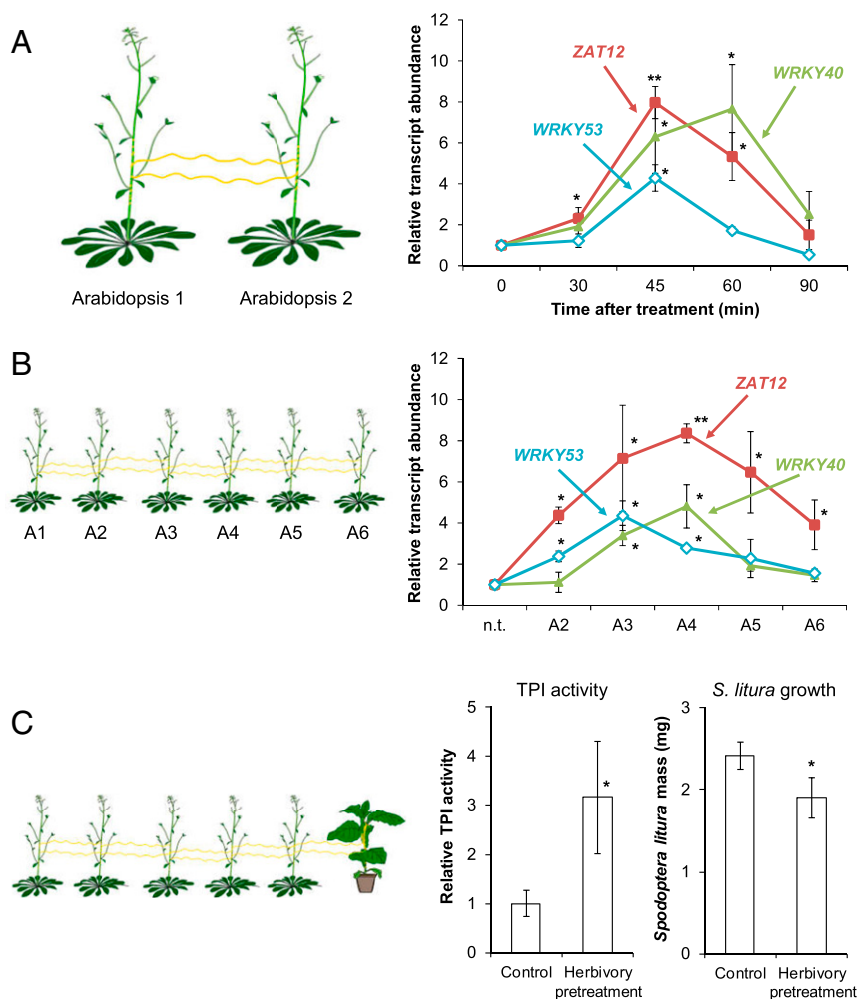
Thus, the *Cuscuta*-mediated interplant systemic signaling occurs rapidly and can induce defense responses even in distantly connected hosts.

## Discussion

*Cuscuta* parasitism influences host physiology and affects host-insect interactions, as *Cuscuta* draws water and nutrients from hosts and its parasitization likely influences host JA and/or salicylic acid levels, leading to changes in defense against insects; for example, *Cuscuta*-parasitized tomato exhibited increased resistance to the beet armyworm *Spodoptera exigua* (26, 27). In the present study, by using *Cuscuta* to transmit systemic signals, we studied how insect herbivory on one host plant affects the defenses of the other hosts in the same *Cuscuta*-connected plant



**Fig. 3.** Insect OS amplify wounding-induced levels of *TPI* in *Cuscuta* bridge-connected systemic tobacco plants. (Left) Schematic of paired *C. australis*-connected tobacco plants. Tobacco 1 was not treated (n.t.) or wounded with a pattern wheel, and 40  $\mu$ L of water (W+W) or *S. litura* OS (W+OS) were immediately applied to the wounds; samples from Tobacco 2 were harvested after 8 h and the *TPI* transcript levels were determined (Right). The nontreated (n.t.) group served as controls, and their values are normalized to 1. Values are means  $\pm$  SE, and asterisks indicate significant differences ( $n = 5$ ; \* $P < 0.05$  and \*\* $P < 0.01$ , *t* test).



**Fig. 4.** *C. australis* mediates rapid and far-reaching wounding- and herbivory-induced systemic signaling. (A) Wounding-induced responses in the systemic undamaged *Arabidopsis* in *C. australis*-connected *Arabidopsis* plant pairs. (Left) Schematic of *C. australis*-connected *Arabidopsis* pairs. *Arabidopsis* 1 was wounded with a pair of forceps, and the transcriptional responses of marker genes at the indicated times in *Arabidopsis* 2 were determined (Right). (B) Wounding-induced systemic responses in multiple *Arabidopsis* plants consecutively connected by *C. australis*. Schematic of six *Arabidopsis* plants (A1–A6) in a row pairwise connected by five *C. australis* plants (Left). The first plant (A1) was wounded or not treated (n.t.), and the relative transcript levels of ZAT12, WRKY40, and WRKY53 in the A2–A6 plants were quantified 90 min after wounding (Right). Values from the group not treated are normalized to 1 ( $n = 5$ ). (C) Herbivory-induced systemic responses in tobacco sequentially connected to five *Arabidopsis* plants. The first *Arabidopsis* plant was infested with *S. litura* for 48 h (herbivory pretreatment) or not treated (control). Tobacco leaves were harvested for determination of TPI activity ( $n = 6$ ) or infested with *S. litura* ( $n = 25$ ) and, after 72 h of feeding, the masses of these insects were determined. Values are average  $\pm$  SE, and asterisks indicate significant differences between plants of the control group and the pretreatment group (\* $P < 0.05$  and \*\* $P < 0.01$ , *t* test).

cluster: *Cuscuta* bridges mediate rapid and far-reaching signaling between con- or heterospecific host plants after wounding/insect herbivory, eliciting changes in the transcriptome and PI activity, and, importantly, systemic signaling primes the undamaged *Cuscuta*-connected plants against subsequent insect attack in a largely JA-dependent manner.

*S. litura* feeding on soybean leaves in soybean–*C. australis*–soybean plant clusters specifically regulated 703, 362, and 353 genes in the L, S1, and S2 leaves, respectively, and these leaves had only a relatively small number of the differentially regulated genes in common (Fig. 1). Variations in systemic responses were also observed in *Arabidopsis* plants expressing a  $Ca^{2+}$  reporter, aequorin, whereby wounding or *Spodoptera littoralis* feeding induced different strengths of  $Ca^{2+}$  signals in different leaves (28). In *N. attenuata*, simulated *Manduca sexta* feeding elicited distinct levels of phytohormones, activity of MAPKs, and TPI in different systemic leaves (23). A metabolomics analysis indicated that largely different metabolites accumulated in local and systemic

rice leaves after *Spodoptera frugiperda* feeding (29). We infer that the underlying mechanism of the differences in systemic responses in a plant or in a *Cuscuta*-connected plant cluster is related to the developmental stages of individual parts, the arrangement of vasculature (i.e., phyllotaxis), and the strength of the systemic signals at a given location, as other researchers have discovered in studying within-plant systemic responses (30, 31).

After wounding, the levels of PI-I increased approximately 1- to 10-fold in systemic tomato leaves (6) and simulated *Manduca sexta* feeding on *Nicotiana attenuata* resulted in 1- to 3-fold increase of TPI activity in systemic leaves (23). These intraplant systemic responses of PIs are similar to what we observed in the *Cuscuta*-connected systemic tobacco plants (Figs. 2B and 4C). In soybeans, we also detected similarly enhanced TPI levels in the S1 and S2 leaves (Fig. S1 and Dataset S1). These findings suggest that the levels of *Cuscuta*-mediated interplant systemic defenses are likely to be similar to those of the defenses induced in intraplant systemic leaves. The effect sizes, quantified in terms of the relatively

decreased masses of the insects growing on *Cuscuta*-bridged systemic plants, were 16% (two *Cuscuta*-connected soybean plants; Fig. 1B), 32% (*Cuscuta*-connected *Arabidopsis* and tobacco; Fig. 2C), 20% (five *Cuscuta*-connected *Arabidopsis* and one tobacco plant; Fig. 4C), and 27% (*Cuscuta*-connected *Arabidopsis* and tobacco; Fig. S5). In some plant communities, herbivore-induced plant volatiles (HIPVs) induce defense responses in the neighboring plants (21). A meta-analysis indicated the effect size to be approximately 0.5, as estimated from 48 studies on HIPV-induced plant resistance, although most studies measured only plant damages as a response to volatile cues (32). Underground common mycelial networks (CMNs) can also act as conduits for signaling between plants: when connected by a CMN, *S. litura* feeding on a tomato plant increased the expression of defense-related genes in the other tomato, and approximately 20% decreased insect masses were detected in the systemic tomato (33). Thus, *Cuscuta*-, HIPV-, and CMN-mediated interplant systemic signaling are all involved in shaping the interactions between plants and insects in different communities, and more research is needed to further demonstrate the ecological role of *Cuscuta*-mediated interplant signaling in nature.

After wounding or insect herbivory, *Cuscuta* transfers certain mobile signals between con- and heterospecific hosts, including hosts from different families. The mobile signals are very likely to be the long-sought wounding/herbivory-induced systemic signals, and, concluding from our data, they are well conserved. We propose that herbivory rapidly elicits these conserved mobile signals in the local leaves, from where they travel through the plant vasculature and induce defense-related responses in other parts of the attacked plant; while moving through *Cuscuta* vines, they activate responses in *Cuscuta* and reach other hosts to induce their defenses. Notably, the systemic signaling regulates only a small number of genes in *Cuscuta*, most of which are related to the primary metabolism, suggesting that the parasites are likely only a physical conduit; nevertheless, it cannot be ruled out that the induced defense responses in the receiver host plants are influenced by interactions of the translocated signal molecules with the parasites (e.g., the translocated signaling molecules may activate certain pathways in the parasites that synthesize more of the same or some other signals).

The nature of the mobile signals and their means of propagation remain largely unknown. Studies on tomato, wild tobacco *N. attenuata*, and *Arabidopsis* have indicated the involvement of JA in systemic signaling (7, 8, 23). However, several lines of evidence support the involvement of other signals: (i) systemic responses, such as activation of MAPKs, were detected in *N. attenuata*, even though the local leaves were excised just 10 min after they were treated with simulated insect herbivory, when JA levels hardly increased in the treated leaves (23), and, similarly, *Arabidopsis* activates JA accumulation in systemic leaves just 2 min after wounding (8, 34); and (ii) electric, hydraulic signals, and ROS are also important parts of systemic signaling (7–9, 11, 12). Importantly, here we show that, compared with the WT *Arabidopsis* plants, wounding *dde2-2* resulted in substantially fewer transcriptional changes in *Cuscuta*-connected tobacco (Fig. 2D), suggesting that JA itself or certain signaling elements regulated by JA are major components of the systemic signals. The findings from this naturally occurring host–parasite–host grafting system are consistent with the results from an elegant artificial grafting experiment: induction of *PI-II* in tomato systemic leaves requires the JA biosynthesis pathway in wounded local leaves (7). Furthermore, analysis of the RNA-seq data revealed more than 400 DEGs in the *dde2-2*-connected tobacco plants, 115 of which are likely not dependent on the JA pathway in the signal sender plants. Thus, other signals are also involved in the *Cuscuta*-mediated systemic signaling, and this is consistent with the fact that the induction of systemic PI activity in *N. attenuata* and tomato is independent of JA accumulation in systemic leaves (7, 23).

Plant vasculatures also transmit other types of long-distance signals induced by biotic stresses, such as attack from pathogens (35) and abiotic stresses, including soil water and nutrient deficiencies (36–38). It is likely that *Cuscuta* could also facilitate multiple stress-induced signaling among different hosts, reshaping the ecology of *Cuscuta*-infected plant communities. By using mutants and WT plants as hosts, this natural grafting system also provides an opportunity to study the role of different signals and signaling pathways elicited by biotic and abiotic stresses.

Studies on human parasites have indicated that, although parasitism generally weakens the human hosts, in some cases, parasitism can provide certain benefits. For example, women infected with roundworms generally have earlier first births and shortened interbirth intervals, increasing fertility (39), and helminth infections often decrease autoimmunity and allergy in humans (40). Plant parasites decrease the fitness of hosts by exploiting host resources, such as water and nutrients. In plant clusters, *Cuscuta* bridge connections enable information exchange among hosts, and, in this way, *Cuscuta* parasitism could alleviate resource-based fitness costs by providing information-based benefits to their hosts. *Cuscuta* may also benefit from this relationship, given that better defended and prepared hosts could provide *Cuscuta* with more nutrients than undefended or naïve hosts in the face of a rapidly dispersing herbivore. As *Cuscuta* spp. are generalists and did not specifically coevolve with any host plant taxa, these are unlikely to be coevolved responses, but likely the simple consequence of vascular fusion between *Cuscuta* and hosts, which allows systemic signals to pass through *Cuscuta* and spread to other hosts in the plant clusters.

## Materials and Methods

**Plant Material and Growth Conditions.** All plants were grown in a glasshouse maintained at ~24 °C (day)/18 °C (night) with a photoperiod of 14 h light and 10 h dark. *C. australis* was cultivated on soybean (*G. max* var. Huachun 6) host plants and had been inbred for six generations. Freshly excised *C. australis* vines from vigorously growing *C. australis*, 10 cm in length, were used to infest new host plants. Approximately 2 wk after initial attachment, new vines emerged and elongated; thereafter, they were allowed to parasitize the neighboring plants. A detailed description of the plant growth and preparation of plant clusters is provided in *SI Materials and Methods*.

**Wounding, W+W, W+OS, and *Spodoptera litura* Feeding Treatment.** *S. litura* eggs were supplied by Genralpest ([www.genralpest.com](http://www.genralpest.com)). For the collection of *S. litura* OS, larvae (third to fifth instar) were reared on tobacco, and OS were collected on ice with a pipette and immediately divided into small aliquots before being stored at –80 °C.

For wounding treatments on *Arabidopsis*, six rosette leaves on each plant were squeeze-damaged with a pair of forceps. For wounding and simulated herbivory on tobacco, four leaves adjacent to the connection sites between hosts and *C. australis* branches that linked the treated plants with the other hosts were wounded with a pattern wheel, and 40 µL of water or *S. litura* OS were gently rubbed into the puncture wounds (W+W and W+OS treatment, respectively).

For herbivory elicitation on soybean, two third-instar *S. litura* larvae were enclosed in a clip cage and three clip cages with a total of six larvae placed on the third trifoliolate leaves (L leaves; Fig. 1) of each one of the paired soybean plants. After 24 h, L leaves, S1 leaves, S2 leaves, and the *C. australis* vines that connected the two soybean plants were harvested for the analysis of herbivory-elicited transcriptomic changes. Plants similarly treated but without insect infestation (empty clip cages) or without *Cuscuta* infestation served as controls. For herbivory elicitation of *Arabidopsis*, three third-instar larvae were placed onto the rosette leaves and were allowed to feed freely for 48 h.

After being harvested, all samples were immediately frozen in liquid nitrogen and stored at –80 °C until use.

***S. litura* Performance Assays.** In *Arabidopsis*–*C. australis*–tobacco plant clusters, *Arabidopsis* was treated with *S. litura* feeding ( $n = 3$  larvae per plant, third instar) for 48 h or left untreated, and *S. litura* insects previously cultivated on Chinese cabbage for 3 d (neonates have a very high mortality on tobacco) were infested on tobacco ( $n = 3$  larvae per plant), and their masses were measured 3 d later.

In soybean–*C. australis*–soybean plant clusters, two third-instar *S. litura* larvae were enclosed in one clip cage and three clip cages with a total of six larvae placed on L leaves of one of the paired soybean plants for 48 h, and freshly hatched *S. litura* larvae were placed in clip cages ( $n = 2$  neonates per cage) on S2 leaves. For comparison, insects were similarly infested on S2 leaves, but without herbivory pretreatment on L leaves (empty clip cages). Three days later, the masses of the larvae were recorded and statistically evaluated.

**Transcriptomic Analyses.** Three biological replicates were used for library constructions and sequencing. Transcriptome sequencing was done at 5 G depth on a HiSeq2500-PE125 platform (Illumina). Each sample yielded between 12 and 18 million high-quality reads. Based on the genome sequences of soybean, *Arabidopsis*, and tobacco, we used TopHat and Cufflinks (41) to assemble the transcripts and to identify DEGs, and genes whose levels were at least twofold changed with statistical significance were selected for further analyses. As the genomes of *Cuscuta* spp. were not publically available, *C. australis* transcriptomes were assembled de novo. We used Trinity (42) with default parameters (k-mer = 25) to assemble contigs and downstream analyses tools of the Trinity software to quantify gene abundances and to identify DEGs. Gene annotation in tobacco and *Cuscuta* were done according to their homology to *Arabidopsis* using Blast software. Analyses of the significantly altered pathways were done by using MetGenMap (20).

**Quantitative Real-Time PCR Analyses.** Quantitative real-time PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad) using iTaq Universal SYBR Green Supermix kits (Bio-Rad). For each analysis, a linear standard curve, threshold cycle number vs. log (designated transcript level), was constructed by using a serial dilution of a specific cDNA standard. The levels of the transcript in all unknown samples were determined according to the standard curves. The *S. pennellii* *ELONGATION FACTOR 1A* (*SpEF1a*), *A. thaliana* *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*AtPP2AA3*), and *N. tabacum* *ELONGATION FACTOR 2* (*NtEF2*), which are all housekeeping genes, were used as the internal controls for normalizing cDNA concentration variations. Primer sequences are listed in Table S3.

**Analyses of Trypsin PI Activity and GS Levels.** TPI activity was analyzed with a radial diffusion assay described by van Dam et al. (43). To quantify GSs, 100 mg leaf material was extracted and analyzed by HPLC (LC-20AD; Shimadzu) as previously described (44).

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