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A species-specific recognition system directs haustorium development in the parasitic plant *Triphysaria* (Scrophulariaceae)

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Abstract. Parasitic plants use host molecules to trigger developmental programs essential for parasitism. One such program governs the initiation, development, and function of haustoria, parasite-specific organs responsible for attachment and invasion of host tissues. Haustoria development can be initiated by several different molecules produced by appropriate host species. We are interested in understanding how these signals are interpreted by two related facultative parasites, *Triphysaria eriantha* (Benth.) Chuang and Heckard, and *T. versicolor* Fischer and C. Meyer, to distinguish their own roots from those of potential hosts. We used an in vitro bioassay to determine what proportion of different *Triphysaria* populations formed haustoria in the presence and absence of closely related and unrelated host species. We found that the proportion of plants with haustoria was the same whether the plants were grown in isolation or with a conspecific host. In contrast, a significantly higher proportion of plants made haustoria when the host was a congeneric *Triphysaria*. Plants with haustoria neither enhanced nor inhibited other plants' propensity to form haustoria. Together these results indicate that qualitative differences exist in haustorium-inducing factors exuded by closely related species. The highest proportion of *Triphysaria* had haustoria when grown with *Arabidopsis thaliana* (L.) Heynh. Even in this case, however, some *Triphysaria* failed to develop haustoria. Interestingly, the percentage of haustoria that had vessel elements was higher when connections were made with *Arabidopsis* than with another *Triphysaria*. These results demonstrate that host recognition can be manifested at multiple points in haustorium development.

Key words: Development – Haustorium – Host recognition – Parasitic plant – *Triphysaria*

Introduction

Self recognition, one of the defining principles of living organism, can take many forms. In plants, the best-studied self-identification systems are those that distinguish pathogens as non-self and those that control sexual compatibility (Nasrallah and Nasrallah 1993; Newbigin et al. 1993; Staskawicz et al. 1995). Parasitic plants have an additional self-recognition system that serves to limit the degree of self parasitism. We are interested in understanding the genetic basis of this recognition system as it is manifested by root parasites of the Scrophulariaceae family.

Plant parasitism is marked by the development of haustoria, parasite-specific organs that attach, invade, and act as physiological conduits for robbing the host plant of water, minerals and carbohydrates (Kuijt 1969; Musselman and Dickison 1975; Riopel and Timko 1995). Within hours after parasite roots come in contact with haustorium-inducing factors the growth and division of cortical cells is altered, resulting in a localized swelling that will develop into a haustorium. In some parasitic species, primary haustoria develop as a transformation of root apices (Kuijt 1969). In others, secondary haustoria develop from parenchymal cortical cells near the root tip as well as at more-proximal positions along the root (Baird and Riopel 1984). Epidermal cells that overlay the swollen root cortex develop long haustorial hairs that function in host attachment (Atsatt and Musselman 1977; Baird and Riopel 1985). Later in haustorium initiation, cortical cells differentiate into vessel elements that form a xylem bridge between the host and parasite (Heide-Jørgensen and Kuijt 1995). Haustorium development is induced in the roots of parasitic plants by molecules exuded from host roots, at least four of which have been isolated and characterized (Lynn et al. 1981; Chang and Lynn 1986; Steffens et al. 1986). In contrast, the development of vessel members requires direct contact with the host tissues (Riopel and Musselman 1979). Therefore, it is likely that at least two different types of host signal are necessary for haustoria to mature.

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Triphysaria is a genus of facultative root parasite in the Scrophulariaceae family (Chuang and Heckard 1991). Like other related parasites, *Triphysaria* has a broad host range and establishes haustorial connections with at least 25 species in 18 different families (Thurman 1966). Even though a large number of host species can be parasitized, only a subset is selected when the parasite is presented with a choice (Atsatt and Strong 1970; Werth and Riopel 1979; Gibson and Watkinson 1991). Host selection presumably allows the parasite to maximize the number of beneficial associations, such as those with nitrogen-rich legumes (Visser et al. 1990). Host selection also allows the parasite to minimize nonproductive associations. A clear illustration of this is the avoidance of self parasitism by most parasitic plants. Some parasites, like *Striga*, completely lack haustoria in the absence of host plants (Okonkwo 1966). Other parasitic plants form haustoria in the absence of host roots but at much lower frequencies than when they are present (Atsatt 1973; Riopel and Musselman 1979). While the biological reasons for limiting self parasitism can be rationalized, the mechanisms underlying this are unknown.

In this manuscript we have characterized self recognition in *Triphysaria*, a small, annual parasitic plant amenable to both classical and molecular genetic analyses. We used an in vitro assay to observe haustorium development in individual *Triphysaria* plants grown both in the presence and absence of host plants. The hosts were either another *Triphysaria* of the same species (conspecific association), another *Triphysaria* of a different species (congeneric association), or *Arabidopsis* (intergeneric association). The haustoria produced in these different associations were examined microscopically to determine whether xylem bridges connecting host and parasite vascular elements were present. Our results show that multiple stages in haustorium development are used as checkpoints for self recognition and host selectivity.

Materials and methods

Materials. Seeds of *Triphysaria versicolor* (Benth.) Chuang and Heckard and *T. erianthus* Fischer and C. Meyer were collected from two different grassland fields in northern California in the late spring of 1994. Seeds were harvested from dried pods and stored at 4 °C. A few plants of each species, together with attached host plants, were dug from the ground and maintained in the greenhouse for manual pollination. Seeds of *Arabidopsis thaliana* (L.) Heynh., Columbia ecotype were obtained from Lehle Seeds (Round Rock, Texas, USA).

In-vitro bioassay for haustorium development. *Triphysaria* and *Arabidopsis* seeds were surface-sterilized by treating for 1 min in 95% ethanol followed by 30 min in 50% bleach and 0.2% Triton X-100. They were then rinsed several times in sterile water to remove bleach. One or two seeds were placed into individual wells of a 24-well tissue culture plate (Corning Inc., Corning, N.Y., USA) containing 1.5 ml of 0.25 × MS (Life Technologies/Gibco-BRL, Gaithersburg, Md., USA), pH 5.8, and 0.8% Noble agar (Difco Laboratories, Detroit, Mich., USA). Plates were then wrapped with Nescofilm to prevent desiccation and incubated at 16 °C in a 12-h light regime under high-output cool-white fluorescent lights (General Electric, Cleveland, Ohio, USA). The roots were visually examined for haustoria each week using a dissecting light microscope (Carl Zeiss, Thornwood, N.Y., USA). These haustoria were almost always connecting two roots, though occasionally unconnected haustoria were observed.

Cytological examination of haustoria for vessel elements. After twelve weeks, haustorial connections were dissected from the in vitro-grown plants and fixed in 10% formaldehyde, 5% acetic acid, and 40% ethyl alcohol (FAA) for at least 24 hours. The roots were then cleared by replacing the FAA with 75% lactic acid and autoclaving for 20 min at 121 °C (O'Brien and McCully 1981). The haustoria were then rinsed in water and observed by light microscopy at 4× to 10× magnification to determined if xylem bridges had formed.

Unattached haustoria were obtained from *Triphysaria* grown in vertically oriented petri dishes containing 0.25 × Hoagland's solution and 1% Phytoagar (Gibco-BRL, New York, USA) after applying 30 μM 2,6-dimethoxybenzoquinone to the roots (Chang and Lynn 1986). Haustoria were identified by the characteristic swelling near the root tip and haustorial hair development. After one week, unattached haustoria were cleared in lactic acid as above and examined for the differentiation of vessel elements.

Results

Recognition of self and non-self in the initiation of haustorium development. The proportion of *Triphysaria* plants that developed haustorial connections in the absence of host roots (autohaustoria) was determined by growing single plants in individual wells of multiwell plates. Similarly, individual *Triphysaria* plants were grown with single *Arabidopsis* Columbia plants. The results are plotted in Fig. 1.

In the absence of host plants, autohaustoria were first observed on *T. versicolor* and *T. erianthus* five and six weeks after sowing, respectively. The percentage of plants with autohaustoria steadily increased until twelve weeks when 21% of the *T. versicolor* and 32% of the *T. erianthus* had autohaustoria. In the presence of *Arabidopsis*, the number of *Triphysaria* with haustoria increased dramatically at all timepoints. More *T. versicolor* had haustoria after three weeks in the presence of *Arabidopsis* than after twelve weeks in the absence of *Arabidopsis*. By twelve weeks, 97% of the *T. versicolor* and 92% of *T. erianthus* had haustoria.

In these and other experiments (data not shown), a higher percentage of *T. versicolor* than *T. erianthus* formed haustoria in response to *Arabidopsis*. Haustoria also developed earlier in *T. versicolor* than in *T. erianthus* (Fig. 1). This suggests that a greater percentage of the *T. versicolor* population than the *T. erianthus* population was sensitive to the *Arabidopsis* inducer. To explore the genetic basis of this difference, we evaluated haustoria formation in F1 hybrids made between *T. versicolor* and *T. erianthus*. F1 seeds were generated by transferring *T. erianthus* pollen to the styles of *T. versicolor*. The hybrid genotype of these seedlings was confirmed from their hybrid phenotypes and from the complete absence of fruit on self-pollinated *T. versicolor* (data not shown). In the presence of *Arabidopsis*, haustorium development

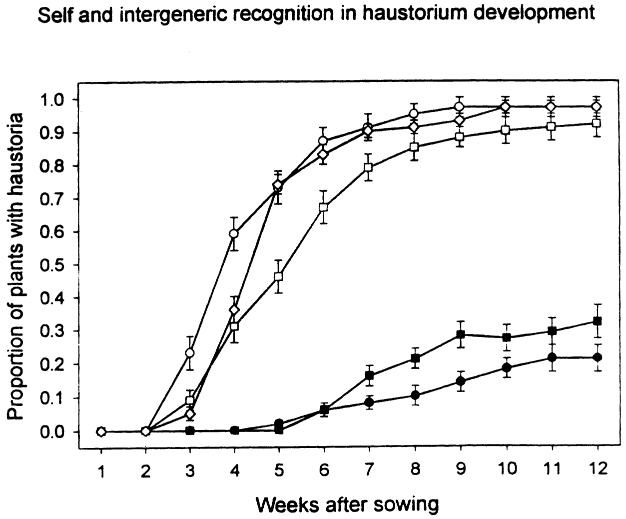

Fig. 1. Self and intergeneric recognition in haustorium differentiation. The proportion of *Triphysaria versicolor* or *T. erianthus* with haustoria when grown in the presence or absence of *Arabidopsis* is shown for each week after sowing. ●–●, *T. versicolor* grown by itself; ■–■, *T. erianthus* grown by itself; ○–○, *T. versicolor* grown with a single *Arabidopsis* seed; □–□, *T. erianthus* grown with a single *Arabidopsis* seed; ◇–◇, F1 hybrids made by crossing *T. versicolor* and *T. erianthus* and grown with *Arabidopsis*. The F1 hybrids were not evaluated in the absence of host plants because of insufficient numbers of seeds. The results plotted were obtained from three experiments. Because one of the experiments was terminated after nine weeks and occasionally wells became contaminated, the total number of plants scored at each week differed. The average number of plants scored for *T. versicolor* in the absence of *Arabidopsis* was 133; for *T. erianthus* in the absence of *Arabidopsis*, 134; for *T. versicolor* in the presence of *Arabidopsis*, 74; for *T. erianthus* in the presence of *Arabidopsis*, 90; and for the F1 in the presence of *Arabidopsis*, 147. Data are means ±SE

in the F1 population most closely mimicked that of the *T. versicolor* parent (Fig. 1).

Autohaustoria formation has genetically inherited components. In order to determine whether autohaustoria were limited to a subset of the population, we examined autohaustoria development in the progeny of selected individuals. Because these species are self incompatible, it was necessary to cross-pollinate plants of similar phenotypes in order to generate progeny. Plants were first classified as to whether or not they had autohaustoria twelve weeks after sowing. These were transplanted to soil and crosses were made among individuals with autohaustoria and among those without. Sufficient numbers of seed for progeny analysis were obtained from eight crosses between parents without autohaustoria and from two crosses between parents with autohaustoria.

The F1 seedlings were evaluated for autohaustoria in the absence of host plants (Table 1). Autohaustoria were observed in all F1 families regardless of whether or not the parents had autohaustoria so the ability to make autohaustoria was not restricted to a subset of the population. The percentage of progeny with autohaustoria did, however, differ between parental classes.

Table 1. Inheritance of autohaustoria formation

Pedigree	Autohaustoria in parents[a]	Number of F1 plants examined	Proportion with autohaustoria[b]
1969	no	46	0.43 ± 0.07
1970	no	46	0.17 ± 0.06
1971	no	24	0.35 ± 0.10
1972	no	24	0.33 ± 0.10
1973	no	24	0.25 ± 0.09
1974	no	43	0.21 ± 0.06
1975	no	23	0.13 ± 0.07
1976	no	22	0.45 ± 0.11
1993	yes	24	0.67 ± 0.10
1994	yes	22	0.59 ± 0.11

[a] Parents in each cross were classified as either making autohaustoria or not by twelve weeks. For pedigree # 1994, only one of the parents had autohaustoria. All the parents were *T. versicolor*.
[b] The proportion of plants with autohaustoria is shown for 10 F1 families 12 weeks after sowing. The SE for each value is determined by $\sqrt{\frac{p(1-p)}{n-1}}$ (Zar 1996)

When the parents had no autohaustoria, the percentages of progeny with autohaustoria ranged from 13% to 45%. However, when at least one of the parents had autohaustoria the percentages of progeny with autohaustoria increased to 59%–67%. This suggests that the ability to form autohaustoria has genetically inherited components.

Haustoria formation in conspecific and congeneric associations. We next determined the proportion of plants that formed haustoria in the presence of a second plant from the same or different *Triphysaria* species. In order to determine which species made the haustoria in congeneric associations, the *T. erianthus* and *T. versicolor* seeds were placed in known locations in the wells.

For both *T. versicolor* and *T. erianthus*, there were no significant differences between the number of plants that formed autohaustoria and the number that formed haustoria in the presence of a conspecific neighbor (Figs. 2, 3). This means that different individuals within a species were not recognized as hosts. There was, however, a striking increase in the proportion of plants with haustoria when the neighbor was a congeneric species. The proportion of *T. erianthus* with haustoria was significantly higher at all times in the presence of *T. versicolor* than in the presence of another *T. erianthus*. Similarly, *T. versicolor* had more haustoria when grown with *T. erianthus* than when grown with *T. versicolor*.

The ability of a plant to form haustoria was independent of whether or not the neighboring plant had haustoria. We used the Poisson binomial to estimate the number of wells that would be expected to have none, one or two plants with haustoria if the plants with haustoria were randomly distributed among the wells (Zar 1996). A non-random distribution was predicted if the presence of haustoria on one plant either enhanced

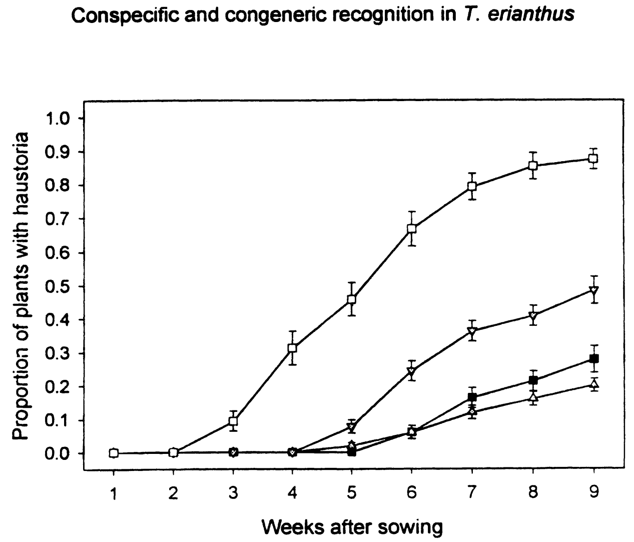

Fig. 2. Conspecific and congeneric recognition in *T. erianthus*. △–△, Proportion of *T. erianthus* with haustoria when two *T. erianthus* plants were grown together; ▽–▽, proportion of *T. erianthus* with haustoria in the presence of *T. versicolor*. The results plotted are from two experiments. The average number of *T. erianthus* plants scored per week was 368 in the presence of a second *T. erianthus* and 177 in the presence of *T. versicolor*. The data for autohaustoria and for the *Arabidopsis* associations are the same as in Fig. 1. ■–■, *T. erianthus* autohaustoria; □–□, haustoria of *T. erianthus* grown with *Arabidopsis*. Data are means ±SE

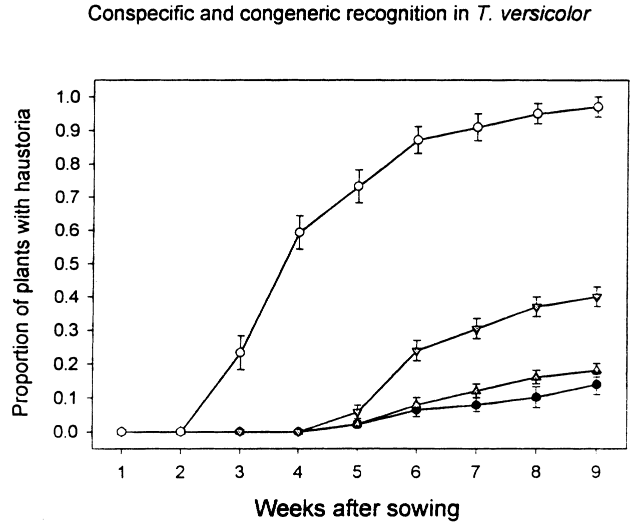

Fig. 3. Conspecific and congeneric recognition in *T. versicolor*. Figure 3 is similar to Fig. 2 but shows the proportion of *T. versicolor* plants with haustoria when grown with either with *T. versicolor* (△–△) or *T. erianthus* (▽–▽). The number of *T. versicolor* scored with a second *T. versicolor* was 354 and with *T. erianthus* 177. As in Fig. 2, the proportion of plants that make autohaustoria and the proportion that make haustoria in response to *Arabidopsis* are the same as used in Fig. 1. ●–●, *T. versicolor* grown without a second plant; ○–○, *T. versicolor* grown with *Arabidopsis*. Data are means ±SE

Table 2. Plants with haustoria are randomly distributed among wells

	Plants in each well		
	Two *T. versicolor*	Two *T. erianthus*	One *T. versicolor* and one *T. erianthus*
Total plants[a]	296	316	400
Plants with haustoria	48	52	157
Total wells	148	158	200
Number of wells in which neither plant has haustoria	100	115	86
Number expected[b]	107	114	91
Number of wells in which one plant has haustoria	41	34	71
Number expected[c]	35	37	72
Number of wells in which both plants have haustoria	7	9	43
Number expected[d]	6	7	37

[a] The total numbers of plants and the numbers of plants with haustoria for three different associations are shown for eight weeks after sowing. The total number of wells is also shown. In all cases there were two plants per well

[b] The expected frequency of a well having no plants with haustoria is $e^{-\mu}$ where μ is the mean number of plants with haustoria per well(Zar 1996)

[c] The expected frequency of a well having only one plant with haustoria is $e^{-\mu}\mu$

[d] The expected number of wells in which both plants have haustoria is the total number of wells minus the number in which one or none of the plants have haustoria

or inhibited haustoria formation on the second. However in no case did the distribution of plants with haustoria deviate from randomness (Table 2).

Post contact differentiation in intraplant, conspecific, congeneric, and intergeneric associations. To see whether *Triphysaria* haustoria required direct contact with host tissue in order to form xylem bridges, haustoria were induced on individual roots of *T. versicolor* with 2,6-dimethoxybenzoquinone. Unattached haustoria were recovered two weeks later and cleared in lactic acid. We found no evidence of xylem formation in 98 unattached haustoria examined.

We also evaluated xylem bridge formation in haustoria that developed in different host-parasite associations (Table 3). Between 30% and 43% of the haustoria had vessel elements in *Triphysaria-Triphysaria* associations. There were no differences in the proportion of xylem bridges when the haustorial connections were between different roots of the same plant or between different *Triphysaria* plants. However, when the *Triphysaria* was attached to *Arabidopsis*, 65–84% of the haustoria had xylem bridges. This indicates that xylem differentiation can discriminate between hosts.

Table 3. Frequency of xylem bridges generated in intraplant, conspecific, congeneric, and intergeneric associations

Plant partners[a]	# haustoria examined	# haustoria with xylem bridges	Proportion of haustoria with xylem bridges[b]
Unattached *T. versicolor* haustoria	98	0	0
T. erianthus, solo	52	21	0.40 ± 0.07
T. versicolor, solo	83	36	0.43 ± 0.05
T. erianthus with *T. erianthus*	80	24	0.30 ± 0.05
T. versicolor with *T. versicolor*	122	43	0.35 ± 0.04
T. versicolor with *T. erianthus*	133	42	0.32 ± 0.04
T. erianthus with *Arabidopsis*	48	31	0.65 ± 0.07
T. versicolor with *Arabidopsis*	37	31	0.84 ± 0.06

[a] Unattached haustoria were induced in *T. versicolor* by 2,6-dimethoxybenzoquinone in the absence of root-root contacts. Plants labeled "solo" were grown by themselves and the haustoria that formed were between roots of the same plant. When haustoria were formed in associations between *T. versicolor* and *T. erianthus*, we did not distinguish which species made the haustoria

[b] The percentage of haustoria with xylem bridges and the SE are shown for each interaction

Discussion

The facultative parasitic Scrophulariaceae typically have a broad host range and form haustorial connections with many host species (Piehl 1963; Kuijt 1969; Gibson and Watkinson 1989). However not all hosts are equally preferred (Atsatt and Strong 1970; Werth and Riopel 1979; Gibson and Watkinson 1991). For example, legumes are often favored because the parasite's mineral requirements are met in part through connections with the host (Visser et al. 1990; Seel et al. 1993; Press 1995). Reciprocally, host selectivity minimizes non-productive associations, such as those between roots of the same individual or between roots of closely related individuals. Even though these associations are undesirable, they will be among those most frequently encountered in a genus like *Triphysaria* whose seeds are not easily dispersed and whose roots are shallow and fibrous (Heide-Jørgensen and Kuijt 1993).

Parasitic weeds are enormously destructive in managed ecosystems in many parts of the world (Parker and Riches 1993). *Striga*, a close relative of *Triphysaria*, infests over 60% of land under cultivation in sub-Saharan Africa where it is the major biotic restraint to agriculture (Berner et al. 1996). Other xylem-tapping parasites, notably the dwarf mistletoes, are among the most serious forestry pests in the western United States (Parker and Riches 1993). Host resistance can be a significant component of pest management strategies when genetic resistance is available. Understanding how a parasite distinguishes its own roots from a host may provide insight into genetic resistance mechanisms and clues for engineering host resistance.

Autohaustoria formed on about 20–30% of *Triphysaria* in the absence of host plants. The number of plants with autohaustoria increased steadily over time, consistent with the hypothesis that the haustoria develop in response to haustorial inducer factors present in the plant's own exudates. However, if *Triphysaria* roots make haustorium inducing factors, why are there not more roots with autohaustoria? And if several different phytomolecules act as haustorium inducing factors, how are those made by *Triphysaria* distinguished from those made by preferred hosts?

The simplest explanation is that *Triphysaria* roots make the same haustorium inducing factors as other plants but just make less of it. We have two lines of evidence that suggest this is not the entire story. For one, the presence of haustoria on one *Triphysaria* did not influence whether the second, neighboring plant had haustoria. This would not be the case if autohaustoria were induced by the increased presence of active molecules. Secondly, both *Triphysaria* species formed haustoria more readily in the presence of a plant from the other species than they did in the presence of a plant from their own. The recognition of congeneric individuals suggests that there are qualitative differences in the exudates from different *Triphysaria* species.

Our experiments do not exclude the possibility that different recognition races exist within a single species. Since the seeds of the two species were collected from different locations, it is possible that what we observed as congeneric recognition results from different recognition races. This offers an alternative explanation for the interesting finding of Riopel and Musselman (1979) that root exudates from *Agalinis* induce haustoria on other *Agalinis*. If the root exudates were made from individuals of a different recognition race, haustoria would be induced. Recognition races can be identified by determining whether different collections of the same species form haustoria on each other.

Another hypothesis for the relative lack of autohaustoria is that the parasite makes haustorium-inducing factors but also makes inhibitors that inactivate their action. Inhibitors of *Striga* haustoria formation have been identified (Smith et al. 1996). However in our experiments, the proportion of plants with haustoria was the same when *Triphysaria* was grown by itself or with a conspecific neighbor. Also, the distribution of plants with and without haustoria was random among the wells. Both of these results argue against inhibitors being responsible for limiting autohaustoria.

A slightly higher proportion of *T. erianthus* had autohaustoria than *T. versicolor*. This is consistent with an earlier observation that the propensity to form autohaustoria differs between species (Riopel 1983). Our data further suggest that even within a species the propensity to make autohaustoria was inherited since more progeny developed autohaustoria when the parents had autohaustoria than when they did not. The ability to make autohaustoria was, however, not restricted to certain subpopulations since progeny with autohaustoria were obtained from parents without autohaustoria. Because of the relatively small number of F1 families we examined, we could not exclude the existence of populations incapable of forming autohaustoria.

A larger proportion of *T. versicolor* than *T. erianthus* formed haustoria in the presence of *Arabidopsis*. Haustoria development in F1 hybrids was similar to that of *T. versicolor*. It is possible that enhanced sensitivity to *Arabidopsis* is genetically dominant and expressed in both the F1 hybrids and the *T. versicolor* parents. Alternatively, since *T. versicolor* was used as the ovulate parent, sensitivity to *Arabidopsis* might be maternally transmitted. Additional crosses are needed to determine the importance of these differences and to identify responsible mechanisms.

Hosts signals also govern later stages in the host-parasite interaction. As in other hemiparasites, xylem elements do not form in *Triphysaria* in the absence of root-root contact. Xylem elements formed as frequently in haustoria between roots of the same plant as in conspecific and congeneric associations. Xylem formation was, however, significantly higher when contact was made to an *Arabidopsis* root. Therefore, recognition of appropriate host species is manifested at late developmental stages as well as early ones.

One hypothesis for the apparent wide host range of parasitic Scrophulariaceae has been that several different races, each specific for a different host, are present in the seed bank. In this regards it is interesting that not all *Triphysaria* formed haustoria even after three months with *Arabidopsis*. It might simply be that three months was insufficient time to induce haustoria in these plants. Alternatively, these might represent a subpopulation of plants incapable of recognizing the *Arabidopsis* Columbia inducer signal. Or, these plants might be unable to develop haustoria or respond to inducer. To address these possibilities, we are now conducting a similar series of experiments on a self-compatible, self-pollinating *Triphysaria* species, *T. pusilla*. This will allow us to characterize the genetic factors governing the inability to recognized *Arabidopsis* as a host.

The induction of haustorium development is an excellent system for studying plant cell organogenesis. Determining how different signal molecules are interpreted by the parasite to distinguish potential host roots remains an open and exciting question.

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