Root-knot nematode chemotaxis is positively regulated by L-galactose sidechains of mucilage carbohydrate rhamnogalacturonan-I

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Root-knot nematodes (RKNs) are plant parasites and major agricultural pests. RKNs are thought to locate hosts through chemotaxis by sensing host-secreted chemoattractants; however, the structures and properties of these attractants are not well understood. Here, we describe a previously unknown RKN attractant from flaxseed mucilage that enhances infection of Arabidopsis and tomato, which resembles the pectic polysaccharide rhamnogalacturonan-I (RG-I). Fucose and galactose sidechains of the purified attractant were found to be required for attractant activity. Furthermore, the disaccharide α-L-galactosyl-1,3-L-rhamnose, which forms the linkage between the RG-I backbone and galactose sidechains of the purified attractant, was sufficient to attract RKN. These results show that the α-L-galactosyl-1,3-L-rhamnose linkage in the purified attractant from flaxseed mucilage is essential for RKN attraction. The present work also suggests that nematodes can detect environmental chemicals with high specificity, such as the presence of chiral centers and hydroxyl groups.

INTRODUCTION

Chemotaxis refers to the movements of organisms along a chemical gradient. This mechanism acts as the foundation for animal behaviors such as foraging, courtship, and predator avoidance and is particularly crucial for parasites searching for a host to infect (1–3). The chemical structures and properties of chemoattractants strongly influence the behaviors of their recipients. In the case of parasites, the chemoattractants are often compounds produced by the host. Therefore, the chemical properties of these attractants can also be informative about the parasite-host specificity (3).

One group of parasites that rely on chemotaxis is the plant-parasitic nematodes. These nematodes are prominent agricultural pests that infect many crop species globally, causing up to billions of U.S. dollars (USD) of damages annually (4). Root-knot nematodes are plant-parasitic nematodes from the genus Meloidogyne, with Meloidogyne incognita (hereafter RKN) being one of the most common. RKNs are obligate parasites that enter plant roots to feed on the host plants. After molting once within the eggs, the RKN second-instar juveniles (J2) hatch and travel through the soil in search of suitable host plants through chemotaxis. Once an appropriate host is found, the J2 enter the root tip and migrate into the vasculature, where they induce the differentiation of vascular cells into an enlarged specialized feeding organ known as a gall or root-knot (5, 6). The RKNs then become fully endophytic, during which they feed on the giant cells within the gall and molt three more times before maturation. Adult females emerge from the gall to lay eggs, from which the next generation of J2 is hatched to seek out new hosts. RKN can infect a wide range of different plant species, including economically important crop plants (7). Nevertheless, RKNs do show some degree of host selectivity, suggesting that RKNs do have mechanisms for host recognition and can alter their behavior accordingly.

The search for RKN chemoattractants has been an ongoing endeavor. Root exudates from soy, tomato, Medicago, and pea are known to attract RKN (3, 8–10). Specifically, plant metabolites including phytohormones (salicylic acid, gibberellic acid, and indole-3-acetic acid), amino acids (arginine and lysine), organic acids (vanillic acid, tannic acid, and lauric acid), and other compounds (organic diamines, mannitol, and flavonoids) have been demonstrated to attract RKN (11–13). However, compounds that are found in most plants are probably not very useful for deciphering RKN’s host specificities because they are more likely to function as general cues that guide RKN to any plants in the vicinity. Currently, only a few parasitic nematode attractants have been shown unambiguously to dictate the nematodes’ host specificity. Furthermore, abiotic factors such as pH, ions, and temperature are known to influence the behavior of parasitic nematodes (14). RKNs were shown to be attracted to cadaverine and other organic diamines (8). Because cadaverine synthesis is known to be stimulated by both biotic and abiotic stresses, it seems likely that the physiological state of the host plant also influences RKN host preference (15, 16). Last, seeds from Arabidopsis (Arabidopsis thaliana) and shepherd’s purse (Capsella bursa-pastoris) have been documented to attract nematodes (17, 18). Currently, it remains difficult to fully explain RKN’s host range and selectivity with the limited repertoire of known RKN attractants. Purification and identification of RKN attractants from plant exudates will help to better understand the mechanism that dictates this RKN host-finding behavior.

Mucilage is an excellent model to study chemicals secreted by plants. In many flowering plants, the seed coat or pericarp secretes a mixture of cell wall polysaccharides during seed development known as mucilage, which absorbs water and extrudes rapidly to encapsulate the seed upon hydration (19–21). This phenomenon of
mucilage synthesis is known as myxodiaspory. The biological functions of mucilage remain under debate; however, mucilage synthesis and composition have been characterized extensively (22, 23). The ability of Arabidopsis seeds to attract RKN indeed depends on the extrusion of mucilage (17). To identify other host plant–derived RKN attractants, we examined RKN behavior toward the mucilage from other myxodiasporic plants. Here, we show that flaxseed mucilage contains RKN attraction activity. The purified attractant from flaxseed mucilage was found to structurally resemble rhamnogalacturonan-I (RG-I), and the l-galactose (l-Gal) sidechains of the purified attractant were shown to be critical for RKN attraction. These findings suggest that polysaccharides may represent a previously unknown class of signaling molecule between plants and soil microorganisms, with sugar chirality and functional groups being important factors.

RESULTS
Flaxseed mucilage contains RKN-attracting activity
Arabidopsis seeds have been shown to attract RKN in a mucilage extrusion–dependent fashion; however, no specific chemoattractant has been isolated from mucilage (17). To identify other mucilage-associated RKN attractants, two other well-characterized myxodiasporic plants, chia (Salvia hispanica) and flaxseed (Linum usitatissimum), were chosen for analysis. Two major flaxseed color variants of brown and gold are commonly cultivated, and mucilage from both flax color variants was tested. Mucilage from both brown and gold flaxseeds alone was sufficient to attract RKN, whereas Arabidopsis and chia seed mucilage did not show attraction activities (Fig. 1A). Whole flaxseeds of both the brown and gold varieties were able to attract RKN (fig. S1, A and B). Furthermore, RKNs were capable of entering the roots of flax seedlings to form galls (fig. S1, C to E). These findings imply that RKNs may be able to parasitize flax plants to propagate and thus have an incentive to target flax for infection.

Next, the behavior of RKN toward flaxseed mucilage was examined at different time points with different mucilage concentrations. RKN began to aggregate around brown flaxseed mucilage in as little as 1 hour after incubation and formed a discernable colony within 4 hours, and the aggregation saturated by around 12 hours after inoculation (Fig. 1B). Factors such as whether RKN metabolize the attractants and whether RKN mediate conspecific signaling upon being attracted can affect when and how attraction reaches saturation. Attraction strength was positively correlated with the amount of brown flaxseed mucilage applied (Fig. 1C) and imbibition time during mucilage extraction (Fig. 1D). Flax mucilage of as little as 0.19 µg/µl was sufficient to mediate detectable attraction (Fig. 1C), a concentration reasonably likely to occur in the soil around flaxseeds.
These results suggest that certain RKN chemoattractants are associated with brown flaxseed mucilage. In comparison, gold flaxseed mucilage appears to be slightly less potent compared to the brown variant in terms of RKN attraction. RKN required a longer time to aggregate around gold flaxseed mucilage and higher mucilage concentrations to reach attraction saturation compared to brown flaxseed mucilage (fig. S1, F to H). However, gold flaxseed mucilage is undoubtedly also capable of attracting RKN, and the seed color, therefore, does not appear to be a major determinant that dictates RKN attractiveness. Because of the attraction to brown flaxseed mucilage being more robust than gold flaxseed, subsequent analyses focused only on brown flaxseed mucilage for simplicity.

**Purified attractant from flaxseed mucilage is likely RG-I**

Because the flaxseed RKN attractant is water soluble, it may be purified from flaxseed mucilage using liquid chromatography. Mucilage components were separated by hydrophobicity via C18 reverse-phase high-performance liquid chromatography (HPLC) and then subsequently by molecular weight via recycle size exclusion chromatography (Fig. 2A). Small aliquots from all fractions were kept after each fractionation step to be independently tested for RKN attraction activity, and only fractions containing attraction activities were used for subsequent analyses. After a methanol (MeOH) wash, compounds with a film-like structure were obtained as the purified attractant, indicating that the purified attractant may consist of polysaccharides.

Seed coat mucilage is typically composed of hydrophilic carbohydrate polymers such as pectin and hemicellulose (24–26). Therefore, the purified attractant is likely to contain carbohydrate polymers similar to other mucilage constituents. To test this hypothesis, the purified attractant was subjected to monosaccharide composition and linkage analyses (Tables 1 and 2). The purified attractant consisted primarily of Fuc, Rha, Gal, and galacturonic acid (GalA). Trace amounts of glucose (Glc), xylose (Xyl), and glucuronic acid (GlcA) were also present in the purified attractant, but arabinose (Ara) and mannose (Man) were undetected (Table 1, untreated purified attractant). In addition, the major linkages present in the

![Fig. 2. Purified RKN attractant was likely flax mucilage RG-I.](image-url)
purified attractant included nonreducing terminal Fuc (Fucp1➔), non-reducing terminal Gal (Galp1➔), O-2-linked Rha (➔2Rhap➔), and O-2,3-linked Rha (➔2,3Rhap1➔) (Table 2, untreated purified attractant). These characteristics are consistent with the structure of RG-I, a class of pectin consisting of an alternating Rha-Gal backbone. In the case of flax mucilage, these backbones are decorated with single Gal or Fuc residue sidechains linked to the C3 position of Rha (Fig. 2B) (27, 28). Only nonreducing terminal Gal and Fuc linkages were detected in the purified attractant, indicating that Gal and Fuc in the purified attractant indeed form single-molecule sidechains (Table 2). Furthermore, Gal levels made up more than half of the Rha levels in the purified attractant. This implies that the majority of Rha in the purified attractant is substituted with a single Gal molecule.

Flaxseed mucilage has the unusual property of containing l-Gal, whereas the abundantly existing Gal molecules in nature are d-Gal (27). Specifically, the l-Gal in flax mucilage was shown to be primarily associated with RG-I, whereas d-Gal residues can be found in the arabinoxylan fraction of flaxseed mucilage (27). d-Gal can also be found in type I arabinogalactan sidechains of RG-I in other plants including Arabidopsis (29). A chiral-specific Gal dehydrogenase (GalDH) was used to examine the chirality of the Gal residues in flaxseed mucilage. In control experiments, GalDH was shown to act on d-Gal and l-Ara, whereas it had no effect on l-Gal (Fig. 2C). GalDH had little effect on the Gal level in flaxseed mucilage, consistent with the notion that flaxseed mucilage is enriched with l-Gal (Fig. 2C) (27). Because the purified attractant structurally resembles RG-I, this also suggests that the Gal residues in the purified attractant are likely to be l-Gal.

RG-I is a common component found in many plant cell wall types, including Arabidopsis mucilage but was not shown to contain RKN attraction activity by itself (Fig. 1A). Therefore, the purified attractant must contain additional moieties that enable it to attract RKN. To identify these moieties, various treatments were performed on flaxseed mucilage to systematically decipher the component required for RKN attraction activity. Neither boiling nor protease treatment was able to significantly affect flaxseed mucilage RKN attraction, suggesting that proteins (such as cell wall glycoproteins) are likely not involved in attraction (fig. S2A). The enzyme cocktail Onozuka cellulase, which targets multiple cell wall polysaccharide linkages, also did not affect flaxseed mucilage RKN attraction (fig. S2A). Last, α-fucosidases were used to treat flaxseed mucilage to determine whether the Fuc sidechains of the purified attractant are required for RKN attraction. Neither of the two α-fucosidases tested from Thermotoga maritima or Homo sapiens significantly affected flaxseed mucilage RKN attraction, suggesting that the Fuc sidechains are likely not essential for RKN attraction (fig. S2B).

Components of flaxseed mucilage RG-I that are commercially available were individually tested for RKN attraction activity. Neither RG backbone, RG-Gal oligosaccharides, Gal, nor Fuc alone was sufficient to attract RKN (fig. S3, A to E). In addition to these components commonly found in RG-I, we also considered the possibility of rhamnan being present in the purified RKN attractant. RG-I backbones are known to consist of alternating Rha-GalA residues; thus, theoretically, the Rha:GalA ratios from RG-I should be close to 1:1. However, the Rha levels in purified attractants consistently appeared higher than GalA (Table 1). Although the GalA levels may have been underestimated because of incomplete hydrolysis (30, 31), it may also be possible that flaxseed RG-I contains nontraditional RG backbones or rhamnan that account for the excess Rha. Currently, only certain algae such as Monostroma nitidum are known to synthesize rhamnan, which consist of α-1,3-linked l-Rha residues with α-1,2-linked sidechains bound by sulfate groups (32, 33). The presence of rhamnan in terrestrial plants has yet to be reported. Nevertheless, we tested the RKN attraction activities of both native and desulfated M. nitidum rhamnan. However, no attraction activities were detected (fig. S3F).

**Gal and Fuc sidechains of the purified attractant are essential for RKN attraction**

Partial acid hydrolysis has been shown to preferentially cleave the RG-I side-chain residues before the Rha-GalA backbone (34). By taking advantage of this property, it is possible to test the importance of sidechains of the purified attractant by specifically removing them. Flax mucilage treated with HCl indeed lost the RKN

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**Table 1. Monosaccharide composition of flax mucilage RKN attractant.** Ratios of Fuc, Rha, Ara, Gal, Glc, Man, Xyl, GalA, and GlcA in purified flax mucilage RKN attractant that was untreated, mock treated, or partially hydrolyzed by HCl. Both mock-treated and HCl-hydrolyzed samples were incubated at 80°C for 16 hours, where mock-treated samples were mixed with double-distilled H2O (ddH2O) and HCl-hydrolyzed samples were mixed with 5 M HCl instead. mol %, mole percent.

| Monosaccharide | Untreated purified attractant (mol %) | Mock-treated purified attractant (mol %) | HCl-hydrolyzed purified attractant (mol %) |
|----------------|-----------------------------------|----------------------------------------|-----------------------------------------|
| Fuc            | 8.8                               | 15.7                                   | 0.0                                     |
| Rha            | 41.8                              | 40.5                                   | 77.0                                    |
| Ara            | 0.0                               | 0.0                                    | 0.0                                     |
| Gal            | 24.4                              | 35.9                                   | 3.5                                     |
| Glc            | 1.3                               | 0.0                                    | 0.0                                     |
| Man            | 0.0                               | 0.0                                    | 0.0                                     |
| Xyl            | 0.3                               | 0.0                                    | 0.0                                     |
| GalA           | 22.8                              | 7.8                                    | 19.5                                    |
| GlcA           | 0.6                               | 0.0                                    | 0.0                                     |
attraction activity compared to mock-treated sample after 16 hours of hydrolysis (Fig. 3A). Similarly, treating the purified attractant with HCl for 16 hours also abolished its RKN-attracting activities (Fig. 3B). Monosaccharide composition and linkage analyses were performed on the purified attractants that had been mock treated or HCl hydrolyzed. As expected, the chemical structure of mock-treated purified attractant resembled that of the untreated attractant (Tables 1 and 2, mock-treated purified attractant). However, Fuc and Gal levels were markedly reduced in the HCl-hydrolyzed purified attractant, and only Rha and GalA remained (Table 1, HCl-hydrolyzed purified attractant). Consistent with this finding, linkage analysis results showed that nonreducing terminal Fuc, nonreducing terminal Gal, and O-2,3-linked Rha linkages were also lost in the HCl-hydrolyzed purified attractant (Table 2, HCl-hydrolyzed purified attractant). These results support the notion that HCl hydrolysis indeed removed only the Fuc and Gal sidechains from the purified attractant, whereas the Rha-GalA backbone remained intact. This implies that the Fuc and/or Gal sidechains in the purified attractant are indeed essential for RKN attraction.

**L-Gal/(α1-3)-L-Rha is sufficient to attract RKN**

To validate the importance of Gal and Fuc sidechains in RKN attraction, synthetic disaccharides were made to mimic the side-chain junctions found in the purified attractant. Disaccharides consisting of two possible purified attractant sidechains, Gal or Fuc, in both D-enantiomers and L-enantiomers, (α1-3)-linked to L-Rha were synthesized to form L-Gal(α1-3)-L-Rha (1), D-Gal(α1-3)-L-Rha (2), L-Fuc(α1-3)-L-Rha (3), and D-Fuc(α1-3)-L-Rha (4) (Fig. 4A). Only (1) showed detectable RKN attraction activity when at least 150 mM was applied (Fig. 4B). The fact that (2) showed markedly lower attraction activity relative to (1) supports the notion that the chirality of Gal is indeed an important factor in attractant recognition. This also supports the finding that most Gal residues in the purified attractant are L-Gal, similar to flax mucilage RG-I (Fig. 2C) (27). Furthermore, (3) did not show detectable attraction activity, although (1) and (3) are essentially identical except for an extra hydroxyl group found in the L-Gal C6 position of (1).

**Flaxseed mucilage positively regulates RKN infection**

Some nematode attractants have been shown to have nematocidal properties, suggesting that these nematode attractants may have evolved as a mechanism to protect plants from infections or secure nutrients for plants (13, 18). To test whether flaxseed mucilage has detrimental effects, RKNs were incubated in flaxseed mucilage overnight before being used to infect tomato seedlings. Mucilage-treated RKN did not appear to display defects in morphology or infection efficiency (fig. S4), suggesting that flaxseed mucilage likely does not have detrimental effects on RKN. To further examine how flaxseed mucilage regulates RKN infection, Arabidopsis and tomato seedlings were dipped in flaxseed mucilage before being exposed to RKN. Mucilage-treated Arabidopsis and tomato seedlings were indeed more sensitive toward RKN infection and produced significantly more galls by 6 to 9 days after inoculation (Fig. 5, A and B).

Last, we aimed to determine whether this flaxseed mucilage–dependent attraction is conserved in other nematode species. To address this question, the behavior of the soybean cyst nematodes (Heterodera glycines), another prominent plant-parasitic nematode, and Caenorhabditis elegans, the model nematode for genetic analyses, was examined in the presence of flaxseed mucilage. Neither H. glycines nor C. elegans showed detectable preference toward flaxseed mucilage (Fig. 5, C and D) (35). This suggests that the flaxseed mucilage–dependent attraction may be a feature limited to parasitic nematodes of the genus Meloidogyne.

**DISCUSSION**

Chemotaxis is a particularly important behavior for parasites, as they are obligated to infect an appropriate host to complete their life cycles. Here, we demonstrate that the L-Gal sidechains of a previously unknown attractant from flaxseed mucilage, which resemble RG-I,
are recognized by RKN to mediate positive chemotaxis. Aside from flax mucilage, several other plant exudates have been documented to interact with nematodes. Seeds from both Arabidopsis and C. bursa-pastoris have been documented to attract nematodes (17, 18). Arabidopsis, mucilage appears to facilitate RKN infection, whereas C. bursa-pastoris mucilage traps nematodes as a form of protocarnivory (17, 18). Root exudates from several plant species also attract nematodes. Lauric acid from crown daisy (Chrysanthemum coronarium) can be nematocidic when presented in certain concentrations, while the root cap exudates from several plant species can immobilize nematodes in a reversible fashion (13, 36). This suggests that plant exudates are not only used by nematodes for host finding but also may be a mechanism to protect plants from pathogens or even capture prey.

Given that mucilage is physically located between plant seeds and soil, it is highly probable that mucilage may be involved in signaling with other soil organisms. Arabidopsis seeds attract RKN in a mucilage extrusion–dependent fashion, suggesting the presence of an RKN attractant in Arabidopsis mucilage (17). However, the flaxseed RKN attractant characterized in this study is unlikely to be related to the one associated with Arabidopsis mucilage. First, the flaxseed mucilage alone is sufficient to attract RKN, whereas, although Arabidopsis seeds with mucilage attract RKN, the mucilage alone contains no attraction activity (17). Second, the Arabidopsis attractant may consist partly of proteins, whereas there is no evidence to suggest the flaxseed attractant contains proteinaceous components (fig. S2A) (17). Last, Arabidopsis mucilage RG-I are largely unsubstituted, and the l-Gal(1→3)-l-Rha (I) linkage has not been reported in Arabidopsis mucilage (29). This may explain why Arabidopsis mucilage lacks RKN-attracting activities, although it is also composed primarily of RG-I (17, 24–26).

By assuming that the purified attractant in this study was indeed flaxseed mucilage RG-I, this is in fact quite an unusual candidate as a pathogen chemottractant. l-Gal is relatively rare in nature and is typically not synthesized in great abundance in plants, with the exception of flaxseed mucilage (27). RKNs are promiscuous pathogens and infect many different plant species; however, flax does not appear to be the predominantly preferred host (7). Therefore, l-Gal–substituted RG-I does not appear to be the main determinant of RKN host preference. Nevertheless, it appears that RKN specifically targets linkages with l-Gal but none of its structural homologs such as l-Fuc and d-Gal (Fig. 4). The preference toward Gal, but not Fuc, is further supported by the fact that α-fucosidase digestion did not significantly affect RKN attraction from flaxseed mucilage (fig. S2B). This specific preference for l-Gal suggests that the hydroxyl group at l-Gal C6 and the Gal chiral center are thus features essential for RKN attraction. It is fascinating that the RKN chemoreceptor is sufficiently sensitive to recognize ligands with specific chiral centers and to discern the presence of a single hydroxyl group. It would be interesting to decipher the selection pressure that led to molecular recognition with such specificity.

Nevertheless, the (1) linkage appears to be abundant in flax mucilage RG-I. More than half of the Rha residues in the purified attractant are likely substituted with l-Gal (Tables 1 and 2). In addition, l-Gal has also been detected in N-glycan, RG-II, and xyloglucan from the plant cell wall, although it is unclear whether they are present in a sufficiently high level to mediate signaling (37–39). However, it is thought that plant pathogens may rely on multiple attractants for host targeting; thus, it is feasible for one of the attractants to be of low abundance (3, 14). RKNs have indeed been documented to infect flax plants (40). A closely related parasitic nematode Meloidogyne hapla is better adapted to colder climates and may be a more efficient flax pathogen than M. incognita (41). It would be interesting to test whether the affinity toward l-Gal–substituted RG-I is conserved in M. hapla as well.

It seems likely that (1) represents only the essential structural motif from larger, more potent RKN attractant molecules that are more biologically relevant. Most carbohydrate receptors bind ligands in the micromolar range (42, 43). In comparison, (1)’s detection threshold of 150 mM appears unusually high. In our analysis, l-Gal has been estimated to occur at roughly 350 μM in the initial flax mucilage, much lower than (1)’s detection threshold of 150 mM. It is possible that the l-Gal sidechains cluster within the purified attractant to locally increase the concentrations above the detection
threshold \((44, 45)\). However, a more realistic scenario would be that RKN detects larger molecules containing the \((1)\) linkage with higher affinity. It is thus important to determine the structure of the optimal attractant to better understand the perception mechanism and its cognate RKN chemoreceptor. Pectic polysaccharides with a degree of polymerization of >9 have been shown to be most potent in inducing immune responses \((46–48)\). Furthermore, the RKN attractiveness of organic diamines has been shown to be correlated with the length of their carbon backbones, with only molecules with three to five carbon atoms having attraction activities \((8)\). These lines of evidence suggest that the structures, particularly the degrees of polymerization, of signaling molecules are critical factors that dictate the signal strength for both plants and nematodes.

Currently, no other cell wall carbohydrates have been shown to be direct signals for microorganisms, although they can function as signaling molecules for plants themselves. Carbohydrates have been documented to regulate meristem maintenance and pollen tube guidance \((49–51)\). Furthermore, carbohydrate fragments formed during cell wall remodeling or pathogen attack can relay cell wall status information to the cell. The pectic homogalacturonan breakdown product oligogalacturonan can mediate cell growth and pathogen response upon perception by the WALL-ASSOCIATED KINASE family of receptor kinases \((52–54)\). Other algae-derived sulfated polysaccharides such as carrageenans \([\alpha-D\text{-Gal}(\alpha1-3)\text{-L-Rha} \quad (1)]\), fucans \([\alpha-L\text{-Fuc}(\alpha1-3)\text{-L-Rha} \quad (3)]\), ulvans \([\beta-D\text{-GlcA}(\beta1-4)\text{-L-Rha} \quad (1)]\), and laminarins \([\beta-D\text{-Glc}(\beta1-3)\text{-Glc with } \beta-D\text{-Glc}(\beta1-6)\text{ branches}]\) have also been documented to elicit plant immune responses \((55, 56)\).

The functions of cell wall polysaccharides are not limited for structure and defense but may also function as signaling molecules. RKNs remain as important pests that infect many crop species in multiple regions of the world and account for millions of USD of agricultural losses annually. The discovery of RKN attractants not only helps to decipher the RKN chemotaxis signaling pathway but

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**Fig. 4. l-Gal sidechains in flax mucilage RG-I are essential for RKN attraction.** (A) Chemical structures of disaccharides synthesized to mimic flax RG-I side-chain junctions. (B to E) RKN chemotaxis indexes of the synthetic disaccharides of \((1)\), \((2)\), \((3)\), \((4)\) from 0 to 500 mM. \(n = 3 \pm SD\). (F) Representative nematode behavior toward 150 mM of the synthetic disaccharides. Dashed circles denote locations where samples were applied.
also may have practical uses in agriculture. RKN attractants may be applied in fields to manipulate nematode behaviors and directly RKN away from sensitive crop plants. Technologies that take advantage of nematode chemotaxis for practical applications such as the N-NOSE have already been developed (57). It remains important to determine the structure of the optimal attractant with the (1) linkage to enhance its utility in agriculture and to characterize its binding mechanism and cognate chemoreceptor. On the basis of the findings from this study, flaxseed mucilage RG-I oligosaccharides with l-Gal substituted sidechains may be the optimal attractant, although the ideal length of the RG-I backbone and the degree of l-Gal substitution remain to be elucidated. A range of RG-I oligopeptides with l-Gal substitutions may be synthesized in vitro and tested for attraction. Currently, the main economic value of flax lies within the seed oil and stem fiber, and utilities in mucilage have great potential to add further value to the current flaxseed industry. The discovery of l-Gal–substituted RG-I as an RKN attractant highlights the ability of animals to recognize signaling molecules with specific chiral centers, the presence of single functional groups, and degree of polymerization, suggesting that biotic communications are highly specific and sensitive processes.

MATERIALS AND METHODS

Plant and nematode materials

Arabidopsis (A. thaliana) seeds of the Col-0 ecotype were used. Chia (S. hispanica) seeds were purchased from Kenkoutairiku. Brown flaxseeds (L. usitatissimum) were donated by the Flax Association of Japan, and gold flaxseeds were purchased from Lohas. Tomato (Solanum lycopersicum) seeds were purchased from Kaneko Seeds.

M. incognita was propagated hydroponically and harvested as described (58). Nematodes used to infect Arabidopsis and tomato seedlings were surface-sterilized before inoculation as described (58). C. elegans were provided by K. Yamanaka (Kumamoto University). H. glycines eggs were collected as described (59). After treatment with 1 nM glycinoeclepin A for 5 days, hatched J2s were collected and washed three times with water.

Preparation of RKN attractants and other cell wall polysaccharides

Seed coat mucilage samples were extracted by imbibing seeds with sterile double-distilled H2O (ddH2O) at a ratio of roughly 40 mg of seeds per 800 μl of ddH2O and rotated at room temperature overnight. The seeds were removed, and the mixture was centrifuged at 13 krpm for 10 min to remove impurities. The supernatants were freeze-dried and resuspended in ddH2O to maintain the initial seed:water ratio. To purify the RKN attractant from flaxseed mucilage, mucilage was extracted from 3.69 g of flaxseeds in 50 ml of sterile ddH2O. The supernatant of the mixture was freeze-dried to give the crude materials (222 mg). The crude materials were subjected to medium pressure liquid chromatography over a C18 reversed-phase column (YMC-DispoPack AT ODS-25, 35 mm inner diameter by 115 mm) with stepwise elution (100% H2O, 50% H2O/CH3CN, and 100% CH3CN) at a flow rate of 2.0 ml/min to obtain water-soluble component (100% H2O fraction; 106 mg). The

Fig. 5. Flax mucilage attracted RKN specifically and positively enhanced RKN infection rates. (A and B) Number of galls formed per three flax mucilage–treated or water-treated seedlings during 3, 6, and 9 days post-inoculation (DPI), for Arabidopsis Col-0 (A) and tomato (B) seedlings inoculated with RKN. n ≥ 6 ± SD; *P < 0.05 and **P < 0.01 compared to water-treated seedlings. (C) Chemotaxis indexes of M. incognita (RKN) and H. glycines toward flax mucilage. n = 3 ± SD; *P < 0.05 compared to M. incognita. Bottom panels show representative nematode behavior toward mucilage. Dashed circles denote the locations where samples were applied. (D) Chemotaxis indexes of C. elegans toward flax mucilage and isoamyl alcohol. n = 3 ± SD; ***P < 0.001 compared to isoamyl alcohol.
water-soluble sample was subjected to recycle HPLC over a size exclusion column (Asahipak GS-310 20G, 20 mm inner diameter by 500 mm) with 300 ml of H2O elution at a flow rate of 8.0 ml/min. The eluted attractant was then washed with 250 ml of MeOH to remove impurities of similar molecular weight, as concentrated RG-1 is insoluble in MeOH. Purified attractant (10 mg) was obtained as an amorphous powder.

For partial acid hydrolysis, 900 µl of flaxseed mucilage or purified attractant was mixed with 100 µl of 5 M HCl or ddH2O for mock treatment. Samples were then incubated at 80°C for up to 16 hours. Acid-hydrolyzed samples were then neutralized with NaOH, and all samples were then dialyzed in 3 liters of ddH2O at 4°C overnight using dialysis membrane with a 14-kDa molecular cutoff (Wako, size 8). Dialyzed samples were freeze-dried and resuspended in ddH2O, maintaining the same initial seed:water ratio.

For mucilage sample treatments, 1-ml flax mucilage aliquots were boiled for 10 min and treated with 2 mg of Onozuka RS (Yakult) or 2 mg of Pronase (Wako) in buffer [0.1 M tris (pH 7.5) and 10 mM CaCl2] at 37°C overnight. For β-fucosidase treatments, 50-µl aliquots of flax mucilage were treated with 0.005 U of T. maritima β-fucosidase (Megazyme) in phosphate buffer (100 mM) buffer (pH 5.0) at 95°C or H. sapiens -fucosidase (Megazyme) in sodium acetate buffer (100 mM) (pH 4.0) at 25°C overnight. Preparations of cell wall carbohydrates 1,3(4)-β-D-galactobiose mix (5 mg/ml; Megazyme O-GBI), RG-I oligomer (5 mg/ml; T. Ishii, Forestry and Forest Products Research Institute), and soy RG (5 mg/ml; Megazyme, 20202b) were made in ddH2O.

Native rhamnans from M. nitidum was extracted as described (60). Briefly, dried M. nitidum was treated with 1 M HCl at 60°C for 3 hours and centrifuged. The supernatant was collected, and rhamnan sulfate in the supernatant was precipitated by adding two volumes of ethanol. Desulfated rhamnan was prepared by solvolytic desulfation with 10% MeOH in dimethyl sulfoxide (60, 61).

**Nematode attraction assays**

Attraction assays with RKN and *H. glycines* were performed essentially as described (17). Assays were performed in 60-mm petri dishes using Pluronic F-127–based matrix (36). For each petri dish, 20,000 nematodes were mixed with 3.5 ml of attraction medium [1.5 ml of using Pluronic F-127–based matrix (36)]. For each petri dish, 20,000 nematodes were placed in a petri dish and incubated at 26°C in the dark for 20 hours before observing nematode behavior. Attraction indexes were 9 of 11

Chemotaxis index = \[
\frac{[\text{sum(#attracted)} - \text{sum(#background)}]}{\text{sum(#total)}}
\] (1)

where #attracted and #background refer to the quantity of nematodes that gathered at/near an attractant and the cognate negative control, respectively, and #total is the total quantity of nematodes used. The quantity of nematodes was defined as the number of pixels occupied by nematodes in a 9.5 mm–by–7.2 mm image with the site of attractant or negative control application in the center. Images of the samples were converted into binary, with the saturation level adjusted such that as many of the nematode bodies and as few background artifacts as possible were highlighted. The pixels were counted using ImageJ (17).

Attraction assays with *C. elegans* were performed essentially as described (64). Assays were carried out in petri dishes with chemotaxis medium [1.5% agar, 5 mM phosphate buffer (pH 6.0), 1 mM CaCl2, and 1 mM MgCl2]. As negative controls, 1 µl of attractant and ddH2O was placed on opposing ends of the dish. To immobilize nematodes in the vicinity, 1 µl of 1 M sodium azide was then placed on top of the attractant and negative control. As a positive control, 1% isoamyl alcohol was diluted in ethanol. About 50 to 100 *C. elegans* adults and L4 larvae were gently rinsed off from culture plates with 1.5 ml of S-basal buffer [50 mM potassium phosphate buffer (pH 6.0)] into a glass tube. The nematodes were allowed to settle and were washed twice with S-basal buffer and once with ddH2O. Ten microliters of the *C. elegans* suspension was placed in the center of the plate, which was sealed with Parafilm and incubated at 20°C for 1 hour. The number of nematodes within 1 cm from where the samples were placed was counted. Chemotaxis index was defined as (number of nematodes near the attractant – number of nematodes near the negative control)/(number of nematodes near the attractant + number of nematodes near the negative control).

**Microscopy**

RKN attraction on Pluronic F-127 medium was imaged using a DP74 camera (Olympus) mounted on an Axio Zoom V16 dissecting microscope (Zeiss). Time-lapse images were taken with a Pentax WG-III camera (Ricoh).

To image flaxseed mucilage morphology, one seed was placed in a 24-well plate stained in 0.01% ruthenium red and imaged with a DP74 camera (Olympus) mounted on an Axio Imager M1 compound microscope (Zeiss) at specific time points. To image RKN that entered the flaxseed seedlings, infected seedlings were stained with acid fuchsin as described (65). Stained seedlings were photographed with a DP74 camera (Olympus) mounted on an Axio Zoom V16 dissecting microscope (Zeiss).

**Monosaccharide analyses**

Purified RKN attractant was first hydrolyzed into monosaccharides using 2 M trifluoroacetic acid at 121°C for 1 hour. Sugar composition was determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS5000+ liquid chromatograph (Thermo Fisher Scientific, Tokyo, Japan) fitted with a CarboPac PA-1 column (4 mm by 250 mm) and a pulsed amperometric detector as described (66). To distinguish d-Gal from l-Gal, a mixture of monosaccharides prepared from purified RKN attractant was treated with d-Gal dehydrogenase (GalDH) purchased from Boehringer Mannheim GmbH (Mannheim, Germany) in 50 mM phosphate buffer (pH 8.9) containing 3 mM nicotinamide adenine dinucleotide at 25°C for 27 hours and analyzed by HPAEC-PAD.

**Carbohydrate linkage analyses**

Carbohydrate linkages were determined by gas-liquid chromatography (GLC) with a Shimadzu gas chromatograph GC-8A fitted with a column (0.22 mm by 25 m) of BPX70 (Shimadzu, Kyoto, Japan), as described (67, 68). The purified flax mucilage was permethylated, hydrolyzed with 2 M trifluoroacetic acid for 1 hour at 121°C, and converted to alditol acetates. The methylated alditol acetates were
analyzed on the GLC system. The methylated alditol acetates were identified on the basis of the relative retention times of standards and those reported previously (68).

Nematode infection assay

*Arabidopsis*, tomato, and flaxseeds were surface-sterilized and germinated on medium containing Murashige and Skoog (MS) basal salts (Sigma-Aldrich), 1% (w/v) sucrose (Wako, 196-00015), 0.05% (w/v) MES (pH 5.7), and 1% (w/v) agar for 7 days at 22°C and constant light. Seedlings were then transferred to petri dishes with infection medium [quarter-strength MS medium, 0.5% Phytagel (pH 6.4; Sigma-Aldrich, P8169)], with six seedlings per petri dish and three dishes per replicate. For *Arabidopsis* and tomato seedlings, three seedlings were first dipped in autoclaved flaxseed mucilage, while the other three were dipped in sterile ddH₂O before transferring to infection medium. The seedlings were inoculated with 500 RKN J2 larvae per petri dish (~80 larvae per seedling). The seedlings were incubated upright with their roots covered by black paper at 25°C under a short-day cycle. Galls were counted at 3, 6, and 9 days after inoculation.

Statistical analyses

For nematode attraction assays, the results consist of the means of three technical replicates ± SD. At least two biological replicates were performed with similar results. For the attraction assays of *M. incognita* and *H. glycines*, values from a single petri dish described above (consisting of three 1-μl samples and three 1-μl negative controls) were summed to be considered as one technical replicate. For the attraction assay with *C. elegans*, the value from a single petri dish described above (consisting of one 1-μl sample and one 1-μl negative control) was considered as one technical replicate. Significant differences were calculated using Student’s *t* test, where *P* < 0.05 denotes significance.

For the nematode infection assays, the results consisted of the means of three technical replicates ± SD. At least two biological replicates were performed with similar results. Total gall number from three similarly treated *Arabidopsis* and tomato seedlings from one petri dish was considered as one technical replicate, while total gall number from all six flax seedlings from one petri dish was considered as one technical replicate. Significant differences were calculated using Student’s *t* test, where *P* < 0.05 denotes significance.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/27/eabbb182/DC1

View/request a protocol for this paper from Bio-protocol.

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