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Host factors influence the sex of nematodes parasitizing roots of *Arabidopsis thaliana*

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Abstract

Plant-parasitic cyst nematodes induce hypermetabolic syncytial nurse cells in the roots of their host plants. Syncytia are their only food source. Cyst nematodes are sexually dimorphic, with their differentiation into male or female strongly influenced by host environmental conditions. Under favourable conditions with plenty of nutrients, more females develop, whereas mainly male nematodes develop under adverse conditions such as in resistant plants. Here, we developed and validated a method to predict the sex of beet cyst nematode (*Heterodera schachtii*) during the early stages of its parasitism in the host plant *Arabidopsis thaliana*. We collected root segments containing male-associated syncytia (MAS) or female-associated syncytia (FAS), isolated syncytial cells by laser microdissection, and performed a comparative transcriptome analysis. Genes belonging to categories of defence, nutrient deficiency, and nutrient starvation were over-represented in MAS as compared with FAS. Conversely, gene categories related to metabolism, modification, and biosynthesis of cell walls were over-represented in FAS. We used β-glucuronidase analysis, qRT-PCR, and loss-of-function mutants to characterize FAS- and MAS-specific candidate genes. Our results demonstrate that various plant-based factors, including immune response, nutrient availability, and structural modifications, influence the sexual fate of the cyst nematodes.

KEYWORDS
cyst nematodes, environmental sex determination, plant–nematode interaction, host–plant interaction

1 | INTRODUCTION

Reproduction is the fundamental biological process of life accomplished through either sexually or asexually in multicellular organisms. In sexual reproduction, the mechanisms regulating the sexual diversion leading to the development of primary and secondary sex characters can differ significantly even among the phylogenetically closely related species (Gempe & Beye, 2010). A number of previous studies have reported the genetic and environmental signals regulating the sexual fate in different model species. In contrast to molecular understanding of genetic regulators, the environmental factors involved in sex determination are not well resolved. A huge variation in the modes of reproduction and sexual differentiation are found in the phylum "Nematoda" comprising over 25,000 species (Hodda, 2011; Zhang, 2013). The molecular studies of sex determination in *Caenorhabditis elegans*, which is a free-living nematode, have laid out a

relatively well-detailed pathway of somatic sex determination (Kuwabara & Kimble, 1992; Zarkower, 2006). However, very few studies addressed the genetic or environmental factors influencing the sexual dimorphism in important species of plant-parasitic nematodes.

Cyst nematodes are plant parasites that induce specialized syncytial feeding structures inside the roots of their host plants. Syncytia are the only food source for developing juveniles and adult females. Cyst nematodes are sexually dimorphic with mobile males and sedentary females. Under field conditions, the proportion of male and female nematodes is of fundamental importance. A high number of females usually leads to increased crop damage and heavy soil infestation. High numbers of males usually occur on resistant plants and indicate adverse conditions for the nematodes. The mechanism of sex determination in this group of plant parasitic nematodes is, however, not clearly understood. Therefore, a better understanding of the factors behind sexual differentiation could be helpful in developing methods to exploit plant factors that lead to male formation.

The infective second-stage juveniles of cyst nematodes (J2) invade the roots in the elongation zone behind the root tip. Once inside the root, stylet thrusts, and the release of cell wall-degrading enzymes and other proteins from the nematode's pharyngeal secretory glands facilitate its intracellular movement through different tissue layers of the host, towards the vascular cylinder. Upon reaching the vascular cylinder, the nematode probes single cells to select a suitable initial syncytial cell (ISC; Golinowski, Grundler, & Sobczak, 1996; Sobczak, Golinowski, & Grundler, 1999; Siddique & Grundler, 2018; Holbein et al., 2019; Marhavý et al., 2019). Once the ISC is selected, the nematode becomes sedentary, and a cocktail of secretions is released into the ISC that manipulates plant defence and metabolic pathways leading to the development of syncytium. Subsequently, nematodes develop into males or females over the period of three moults (J3, J4, and adults). The adult male nematodes leave the roots, but the females remain sedentary and produce eggs after fertilization. Finally, the females die, and their bodies turn into egg-protecting cysts. Because of the prolonged sedentary phase required for reproduction, female cyst nematodes require, on average, 29 times more food compared with males (Müller, Rehbock, & Wyss, 1981). Further, female-associated syncytia (FAS) are larger than male-associated syncytia (MAS).

A considerable amount of work has been carried out to clarify the influence of environmental and genetic factors on the sex of cyst nematodes. The results, however, are not all in agreement. The first report on variations in the sex ratio was provided by Molz (1920), who found that the sex of the sugar beet cyst nematode *Heterodera schachtii* is strongly influenced by the physiological state of the host plants, leading to the suggestion of environmental sex determination (ESD) in cyst nematodes. However, contrasting conclusions were drawn a few years later by Sengbusch (1927), who repeated some of Molz's experiments and suggested that the high percentages of males under unfavourable conditions result from a differential death rate of the female larvae. This conclusion was based on his estimations that females require 35 times more food as compared with males. Later, Ellenby (1954) published the results of experiments with *Heterodera rostochiensis* (syn. *Globodera rostochiensis*) on potato roots and argued that if a variation in the sex ratio is due to the differential death rate of females, then the bodies of the deceased females should be present in the roots. Accordingly, he designated all nematodes that were not adult males as females. Under this determination, the proportion of males to females increased strongly with an increased intensity of unfavourable environmental conditions thus reinforcing the ESD view. Adding a single juvenile of *G. rostochiensis* to the host root resulted in an overwhelming majority of females in two independent studies, which was attributed to a decrease in competition for feeding site induction (Den Ouden, 1960; Trudgill, 1967). Grundler, Betka, and Wyss (1991) performed a number of experiments by adding a single juvenile of *H. schachtii* on *Brassica rapa* growing in a nutrient solution containing minerals and various concentrations of sucrose. They also found that the majority of juveniles developed into females under favourable conditions. Although single-juvenile experiments indicated that ESD plays a role in the sexual outcome of cyst nematodes, in the majority of these experiments, the number of males remained rather constant, whereas the number of females fluctuated under different environmental and nutritional conditions.

Transcriptome analysis is often the first step towards identifying genes and pathways that underlie a biological phenomenon. A transcriptome and proteome analysis of FAS induced by *H. schachtii* in *Arabidopsis* roots provided valuable insights into the molecular functioning of syncytium (Hütten et al., 2015; Szakasits et al., 2009). However, it is technically challenging to compare the differences between MAS and FAS with this approach. For one thing, when the morphological features that allow for sex determination become apparent, the nematodes are already sexually differentiated into males and females (Raski, 1950; Wyss, 1992); therefore, it is unlikely that a transcriptome analysis at this time point would provide insights into the host factors that influence sexual differentiation. For another, the isolation of pure syncytial material during the early stages of syncytium development is challenging and laborious.

Here, we established and validated a novel approach to predict the sex of *H. schachtii* juveniles during the early stages of infection when their sexual outcome is not yet apparent (Wyss, 1992). We isolated pure syncytial material via laser capture microdissection (LCM), which allowed us to compare the transcriptomes of potential MAS and FAS and identify sets of genes that are differentially regulated during the early stages of syncytium development. Our subsequent infection assays of knockout mutants corresponding to the differentially regulated genes provided new insights into plant-based factors that influence sexual differentiation of *H. schachtii*.

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions

Arabidopsis thaliana seeds were surface sterilized using 0.7% NaOCl (v/v) for 5 min followed by three successive washings with sterile

water. The sterilized seeds were grown in Petri dishes containing Knop medium supplemented with 2% (w/v) sucrose (Sijmons, Grundler, von Mende, Burrows, & Wyss, 1991). The plates were incubated in growth room at 25°C, with an alternating period of 16 hr of light and 8 hr of dark under sterile conditions.

2.2 | Nematode measurement, prediction, and infection assay

10-day-old plants were infected with approximately 40 freshly hatched J2s of *H. schachtii*. For relative growth, the J2s that invaded and established in the lateral roots at 24 hr after inoculation (hai) were marked with permanent markers on Petri dishes and imaged daily for the next 5 days. Their feeding establishment was defined when a nematode stopped stylet movements. The average size of a nematode was measured as previously described (Siddique et al., 2014). For each experiment, 40–50 nematodes were measured, and the experiments were repeated three times. An empirical curve was drawn across average measurements, and the sex of the nematodes was re-evaluated at 12 days postinfection (dpi). Similarly, for prediction assays, J2s-established feeding sites in lateral roots were marked at 24 hai, and observing their relative development were predicted at 4 and 5 dpi. The only juveniles associated with single syncytium were considered for prediction. The sex of predicted juveniles was confirmed at 12 dpi. For infection assays, the numbers of males and females were counted. Moreover, the average size of females and the average size of syncytia at 14 dpi were measured as described recently (Anwer et al., 2018).

2.3 | Sample collection, processing, and microarray data analysis

Root segments containing 5 dpi putative MAS or FAS were collected in a Farmer's fixative solution on ice. The samples were embedded in an optimum cutting medium (Polyfreez®) using Tissue´Tek® cryo moulds, and sections of 10 μm were cut, and the total RNA was extracted as described previously (Anjam et al., 2016). A cDNA synthesis was performed with NuGEN's Applause 3'-Amp System (Cat. No. 5100), according to the manufacturer's instructions, and started with 100 ng of total RNA. NuGEN's Encore Biotin Module (Cat. No. 4200–12) was used to fragment 3.95 μg cDNA followed by biotin labelling according to the manufacturer's instructions. Hybridization, washing, and scanning were performed according to the Affymetrix 30 GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). Three chips were hybridized for MAS and FAS, with each microarray representing an independent, biological replicate. Primary data analysis was performed with Affymetrix software Expression Console v1.* using the MAS5 algorithm. Statistical analysis of microarray data was performed as described previously (Mendy et al., 2017). The gene set enrichment analysis of FAS and MAS data was performed using PlantGSEA tool kit as described previously (Yi, Du, & Su, 2013). The genes with fold change >1.5 and p value < .05 were selected for enrichment analysis in gene ontology (GO) annotations of domain "biological process." We compared expected and actual percentage of gene enrichment in selected subcategories. The number of *Arabidopsis* genes used for calculating expected enrichment was 27,029. Gene set enrichment analysis used these genes from TAIR database annotated into 7,041 GO gene sets (Yi et al., 2013). The actual enrichment was calculated from 124 FAS and 331 MAS genes. The expression of top 100 FAS and MAS genes in different anatomical parts, under nutrient and biotic stress conditions, was analysed by genevestigator.

2.4 | Genotyping and expression analysis of knockout mutants

Single T-DNA inserted knockout mutants for selected genes (Table S1) were ordered from the relevant stock centre. The homozygosity of SALK mutant lines (NASC, The European Arabidopsis Stock Centre, www.arabidopsis.info) was confirmed via PCR using primers given in Table S2. The homozygous mutants of SALK and GABI-KAT lines (University of Bielefeld, Germany, www.gabi-kat.de) were confirmed to be completely absent for expression of required gene through RT-PCR with gene specific primers given in Table S2.

2.5 | Development of promoter–reporter lines and β-glucuronidase analysis

Promoter regions upstream of the 5' UTR of *LPTG-6* (1,361 bp), *BGLU28* (1,471 bp), and *CWLP-1* (1,214 bp) were amplified by Gateway PCR using *Arabidopsis* Col-0 genomic DNA as template using primer given in Table S3. Subsequently, promoters were cloned via gateway cloning upstream of β-glucuronidase (*GUS*) gene in pMDC162. *Promoter::GUS* constructs were introduced into *Agrobacterium tumefaciens* GV3101 for transformation of *Arabidopsis* Col-0 plants by the floral dip method. T3 homozygous lines were generated and analysed for GUS expression as described recently (Shah et al., 2017). Four plants from each of three transgenic lines per construct were analysed at each time point.

2.6 | Real time PCR

RNA from LCM-derived MAS or FAS was isolated, and cDNA was amplified. Transcriptome abundance for candidate genes was analysed using StepOne Plus Real-Time PCR System (Applied Biosystems, USA) as described recently (Anwer et al., 2018). β-Tubulin and ubiquitin were used as internal controls. As similar results were obtained by both internal controls, therefore, calculations presented in the manuscript are based on one internal control, β-Tubulin. Relative expression was calculated by the Pfaffl's method (Pfaffl, 2001), where the expression of the candidate gene was normalized to the internal control to calculate fold change. Primer sequences for all genes are provided in Table S4.

2.7 | Anatomy and ultrastructure

Arabidopsis Col-0 plants were grown and inoculated aseptically with freshly hatched J2s of *H. schachtii* as described above. Root segments containing putative MAS or FAS were dissected at 5 dpi, processed for light and transmission electron microscopy, and examined as described previously (Golinowski et al., 1996).

3 | RESULTS

3.1 | J2 females grow faster than J2 males

To determine whether there is a difference in growth patterns between male and female J2s, we grew plants in vitro and inoculated them with nematodes. We marked the juveniles that had successfully established the ISC at 24 hai and monitored their growth daily over the following 5 days. We determined the sex of the marked nematodes at 12 dpi and calculated the growth curves for males and females. Until 3 dpi, there was no difference in the average size between male and female juveniles; however, the female juveniles were significantly larger from 4 dpi and grew faster than male juveniles (Figure 1). These results also suggested that it might be possible to predict the sex of a juvenile during the early stages of infection based on the differences in their sizes.

3.2 | The sex of nematodes can be accurately predicted as early as 5 dpi

To assess whether we can predict the sex of J2 nematodes during the early stages of infection, we inoculated the plants with nematodes and marked successfully established nematodes at 24 hai. The development of nematodes was assessed at 5 dpi, and the sex of the nematodes was predicted as male or female. The predicted sex of the juveniles was re-evaluated at 12 dpi, a time point where morphological differences between males and females can be clearly differentiated. We were able to predict the sex of female nematodes at 5 dpi with more than 90% accuracy, whereas the sex of male nematodes was correctly predicted with approximately 85% accuracy. Thus, the sex of the juveniles can be predicted successfully during the initial stage of infection (Table 1 and Figure 2).

3.3 | Structural and cellular differences between FAS and MAS

To investigate the cellular and ultrastructural differences between MAS and FAS, we predicted the sex of the nematodes at 5 dpi and dissected root segments containing syncytium and attached nematodes. The root segments were serially cross-sectioned and analysed through light and transmission electron microscopy. We found that both MAS and FAS

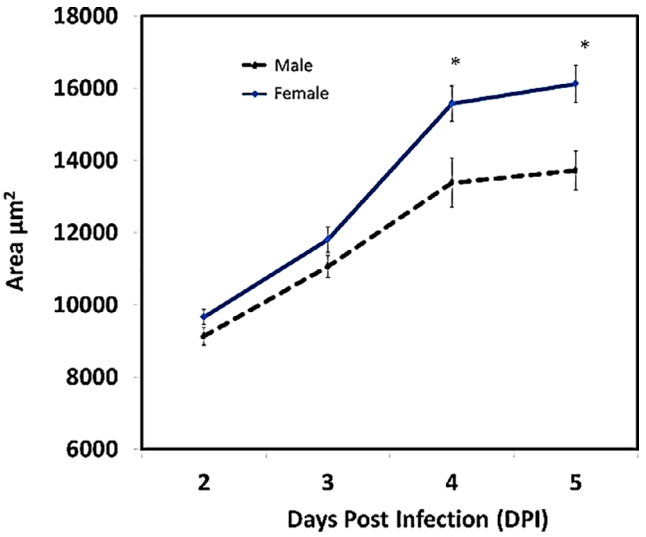

FIGURE 1 Second-stage juveniles of cyst nematodes females grow faster than second-stage juveniles of cyst nematodes males. The nematodes that established an initial syncytial cell in roots were marked, and their growth was monitored over the next 5 days. The values represent the average size of a nematode ± *SE* (n = 54). Data were analysed using two-tailed t test ($p < .05$). Asterisks represent statistically significant differences between size of male and female nematodes at same time point (DPI)

TABLE 1 Sex prediction assays for *Heterodera schachtii*

Nematode	Prediction	Actual	Success (%)
Male	76	66	86.3 ± 1.8
Female	125	115	91.2 ± 1.7

Note: The experiment was repeated three times independently, and percentage of right prediction was calculated. ± sign indicates standard deviation.

expanded via incorporation of hypertrophied vascular cylinder cells, but they differed strongly in anatomy (Figure 3a–j). MAS appear to be composed of fewer cells (Figure 3a–e) than FAS (Figure 3f–j). At the leading edge of the syncytium, where new cells were incorporated into axially expanding syncytium, recently incorporated cells were only slightly hypertrophied in MAS (Figure 3a), whereas their hypertrophy was more pronounced in FAS (Figure 3f). This difference was also clearly recognizable in submedian and median parts of both syncytia (Figure 3b,c vs. Figure 3g,h). In the region close to the tip of the nematode's head where the ISC had been selected, MAS usually had a crescent-like shape (Figure 3d), and many degraded cells were present. By contrast, the FAS were centrally located inside the vascular cylinder (Figure 3i). Below the nematode head, in the juvenile migration region, the extent of damaged cells was very high around predicted male juveniles (Figure 3e), whereas the vascular cylinder was more intact near the predicted female juveniles (Figure 3j). Another anatomical feature differentiating MAS and FAS was the very weak development of periderm-like secondary cover tissue around MAS (Figure 3a–c vs. Figure 3f–i), which consisted of three to four continuous cell layers sheathing the FAS (Figure 3f–h), but only one to two cell layers in MAS (Figure 3a–c).

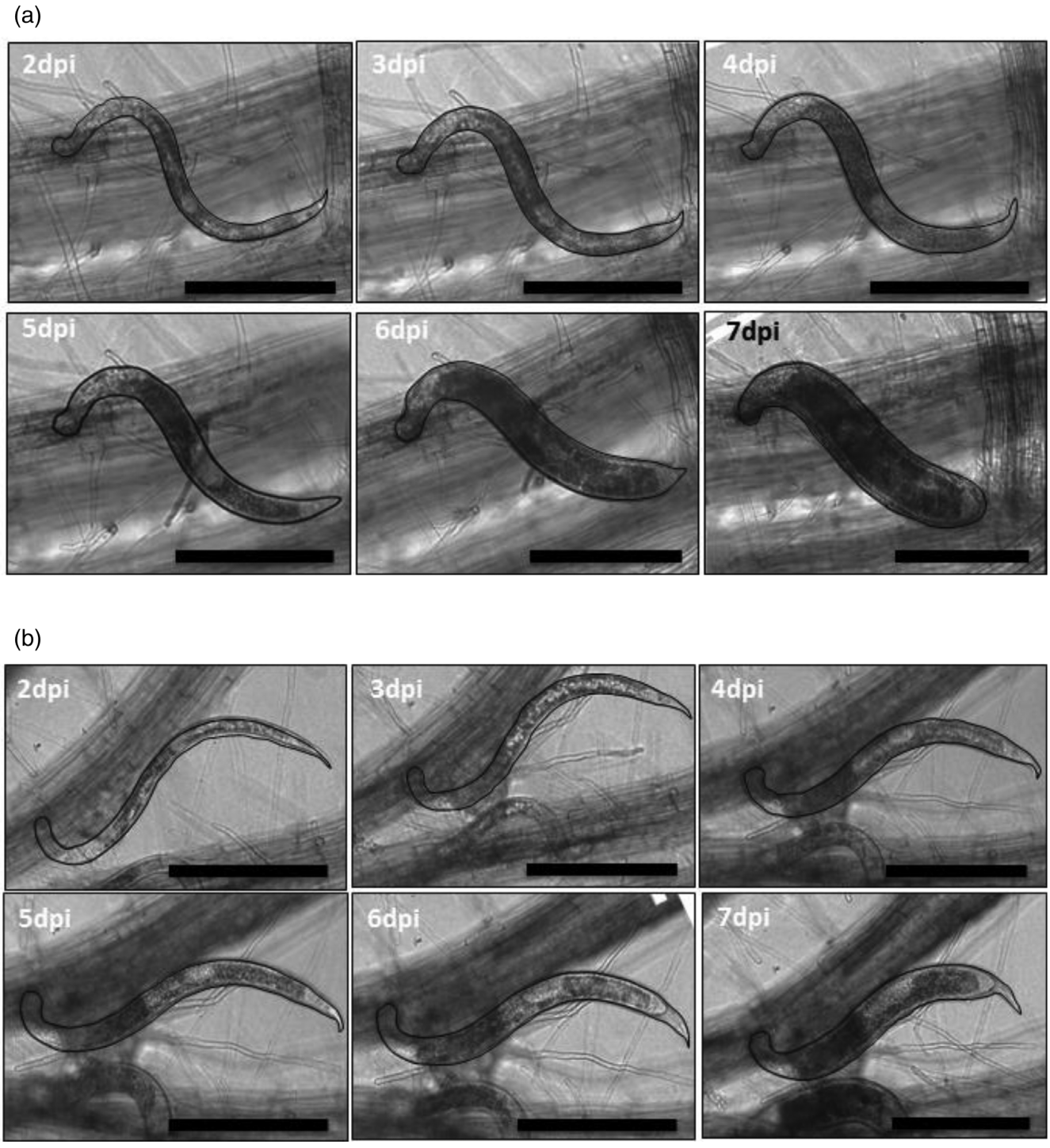

FIGURE 2 The sex of nematodes can be accurately predicted as early as 5 dpi. (a) Development of a female juvenile from 2 to 7 dpi. (b) Development of a male juvenile from 2 to 7 dpi. Note that a line has been drawn around the highlighted nematode to make stand out from other nematodes in the background. Scale bars = 200 μm. dpi, days postinfection

Serial sectioning of syncytia allowed us to localize the heads and stylets of several predicted male and female juveniles. The identification of ISC at this time point (5 dpi) was difficult due to extensive cell enlargement and cell wall openings formation. However, juveniles appeared to always select their ISCs in cells next to the xylem vessels (Figure 3i,k,l). FAS were induced among procambial cells (Figure 3i,k), whereas MAS were induced among pericyclic cells (Figure 3l).

Ultrastructural analysis showed that at 5 dpi; FAS had electron-dense cytoplasm with notably smaller vacuoles (Figure 3m). The protoplasts of FAS contained enlarged and amoeboid nuclei and numerous organelles. By contrast, MAS had less electron-dense cytoplasm almost completely devoid of rough endoplasmic reticulum cisternae, and small vacuoles were more numerous than in FAS (Figure 3n).

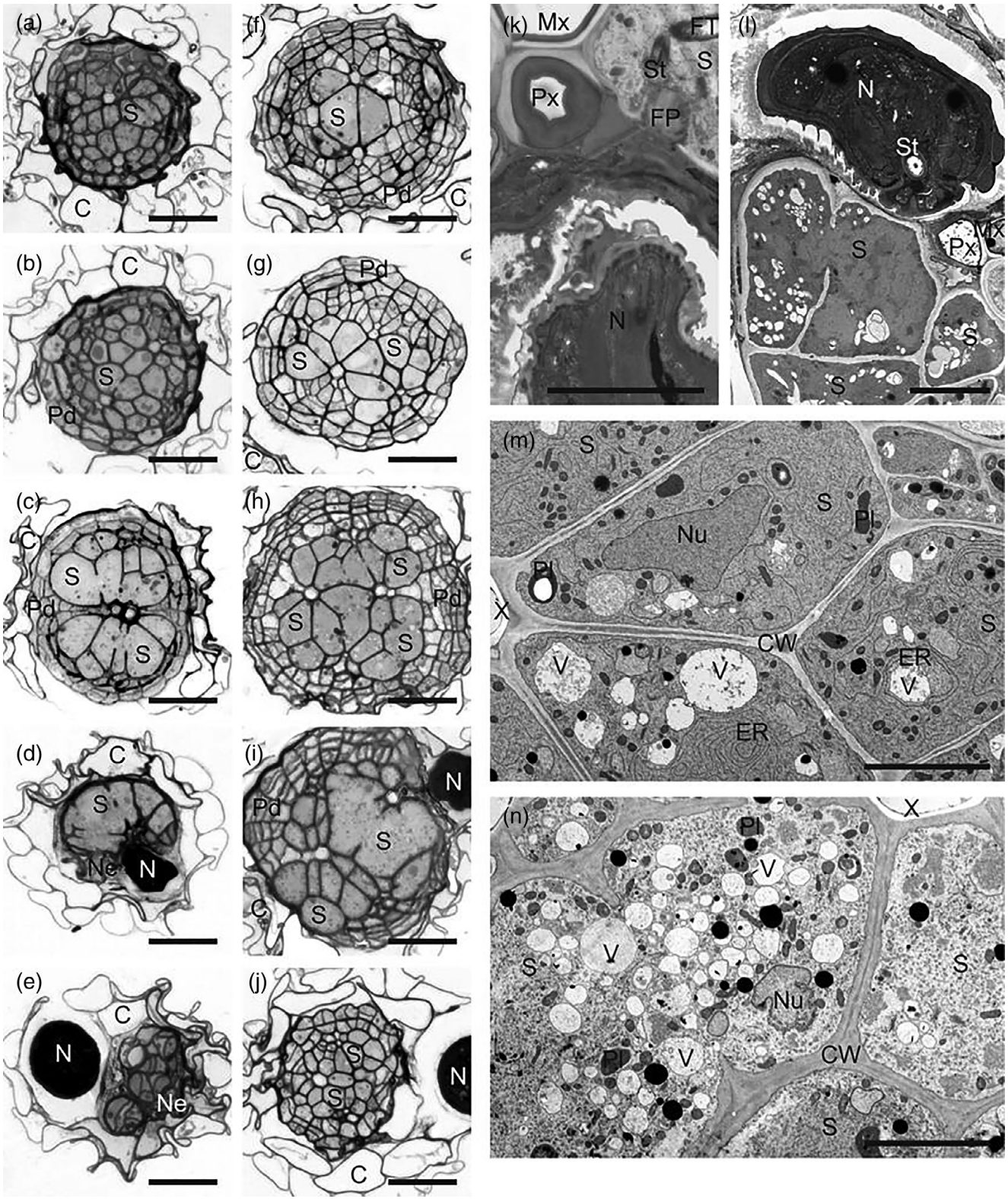

FIGURE 3 Structural and cellular differences are observed between female-associated syncytia and male-associated syncytia at 5 days postinfection. Light (a–j) and transmission electron microscopy (k–n) images taken from cross sections of syncytia associated with male (a–e, l, and n) and female (f–j, k, and m) juveniles. The images were taken from sections made at the leading edge of syncytium (a and f), the submedian region of syncytium (b and g), the median region of syncytium (c, h, m, and n), next to the nematode's head (d, i, k, and l), and along the nematode migration path (e and j). Scale bars = 50 μm (a–j) and 5 μm (k–n). C, cortex; CW, cell wall; ER, cistern of endoplasmic reticulum; FP, feeding plug; FT, feeding tube; Mx, metaxylem vessel; N, nematode; Ne, necrosis; Nu, nucleus, Pd, periderm-like tissue; Pl, plastid; Px, protoxylem vessel; S, syncytium; St, stylet; X, xylem; V, vacuole

3.4 | FAS and MAS have transcriptional differences

To reveal changes in gene expression between FAS and MAS during the early stages of infection, we performed GeneChip analyses. Plants were grown and inoculated with nematodes. Invading J2s that had successfully established the ISC (as defined by a cessation of stylet movements) were marked at 24 hpi, and the sex was predicted at 4 dpi. Root segments containing potential MAS or FAS were dissected at 5 dpi. The samples were embedded in an optimum cutting medium and pure syncytial material was isolated LCM. Total RNA was extracted, labelled, amplified, and hybridized with the *Arabidopsis* ATH1 GeneChip designed for the detection of 24,000 genes. We compared the transcriptomes of FAS and MAS and found that 455 genes were differentially expressed (fold change > 1.5), with a false discovery rate below 5% (Table S5). A higher number of genes showed increased transcript abundance in MAS (331), as compared with FAS (124). FAS- or MAS-specific are used throughout this manuscript to describe genes that have an increased expression in FAS or MAS. We previously showed that changes in cytokinin signalling and biosynthesis strongly influence the sex ratio of cyst nematodes (Siddique et al., 2015). Interestingly, in this work, we found that cytokinin signalling and biosynthesis (*AHK5*, *IPT1*, and *IPT9*) genes were strongly upregulated in FAS as compared with MAS. Lists of the top 50 differentially expressed genes are given in Tables S6 and S7.

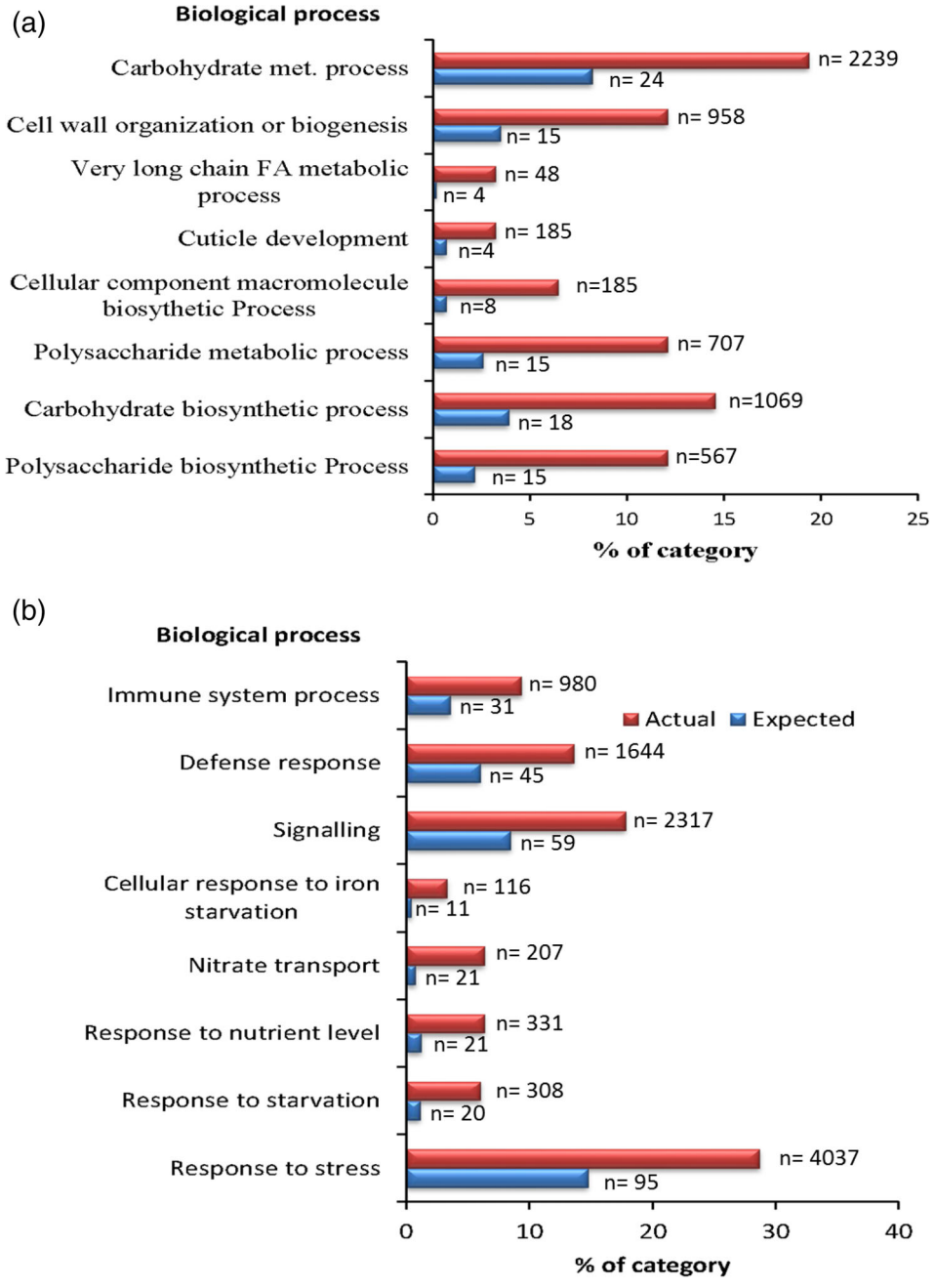

FIGURE 4 Gene ontology enrichment analysis showed overrepresentation of defense- and nutrient stress-related genes in male-associated syncytia. The percentage of expected and actual genes found in the examined subset is shown on the *x*-axis. The blue bar represents the expected set of genes matching the respective category, and the red bar indicates the set of differentially regulated genes in the current study. All gene sets shown have an FDR < 0.05 and a fold change < 1.5. (a) Overrepresentation of female-associated syncytia preferentially upregulated genes (124) with respect to the *Arabidopsis* genome. (b) Overrepresentation of male-associated syncytia preferentially upregulated genes (331) with respect to the *Arabidopsis* genome

3.5 | Defence- and nutrient stress-related genes are over-represented in MAS

To understand the processes that are altered between FAS and MAS, we performed a GO enrichment analysis of FAS- or MAS-specific genes by computing overlaps with 7,041 previously defined data sets using PlantGSEA software (Yi et al., 2013). Categories that were particularly enriched in the genes upregulated in FAS include "polysaccharide biosynthetic processes," "carbohydrate biosynthetic and metabolic processes," "polysaccharide metabolic processes," and many "cell wall-related biosynthetic and metabolic process" categories (Figure 4a). For the MAS-specific genes, categories of "response to stimulus," "response to stresses," "response to starvation," "response to nutrition," and "defence and innate immune response" were significantly over-represented (Figure 4b). The complete list of over-represented categories in GO enrichment for FAS and MAS is given in Tables S8 and S9.

Next, we used Genevestigator (Zimmermann et al., 2014) to analyse the expression of FAS- and MAS-specific genes in response to biotic infections and found that many of the MAS-specific genes were differentially expressed in response to biotic infections (Figure S1), whereas FAS-specific genes showed little to no induction in response to biotic infections (Figure S2). We performed a similar analysis to evaluate the expression patterns of FAS- or MAS-specific genes in response to nutrient stress. Whereas the FAS-specific genes were not related or only slightly related to nutrient stress responses (Figure S3), the MAS-specific genes showed a clear tendency to be induced by iron or sulphur deficiency. Interestingly, the expression of many of the MAS-specific genes was suppressed when seedlings were treated with sucrose or glucose (Figure S4).

3.6 | Validation of GeneChip data by quantitative RT-PCR

The expression of eight FAS- or MAS-specific genes was further validated using quantitative RT-PCR. Our qRT-PCR analysis showed the same trend as that indicated by our microarray analysis (Table 2). However, the fold change for some candidate genes (*CWLP1*, *CELL WALL PLASMA MEMBRANE LINKER PROTEIN*; *GH3.3*, *INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE*; *PGIP1*, *POLYGALACTURONASE INHIBITOR PROTEIN 1*; and *LNG1*, *LONGEFOLIA 1*) was much higher as compared with the microarray results.

3.6.1 | Promoter::GUS analysis

We generated promoter::GUS lines to analyse the spatio-temporal expression of two highly expressed candidate genes, one each for FAS (*CWLP1*) and MAS (*Β-GLUCOSIDASE 28* [*BGLU28*]). One representative homozygous line for each gene was infected with nematodes. The sex of the nematodes was predicted at 5 dpi, and tissues were stained for GUS activity at 5 and 12 dpi (Figure 5).

TABLE 2 Validation of microarray results by qRT-PCR

Gene name	Fold change (female vs. male)	
	Microarrays	qRT-PCR
BGLU-28	−19.75	−12.72 ± 6.4
GH3.3	3.92	8.72 ± 1.74
CWLP-1	13.46	36.25 ± 12.25
PGIP2	−1.86	−2.82 ± 0.97
PDF1.4	−4.33	−1.31 ± 0.25
LTPG6	2.65	2.94 ± 1.24
LNG1	2.58	6.05 ± 1.20

Note: The fold change in expression of candidate genes in female-associated syncytia when compared to male-associated syncytia. The values represent average for three biological replicates ±SE. The (−) indicate upregulation of genes in male-associated syncytia compared with female-associated syncytia.

In *pCWLP1*::*GUS* plants, the majority of the FAS showed moderate to high GUS staining at 5 and 12 dpi, whereas only weak staining was detected in MAS (Figure 5). For the *pBGLU28*::*GUS* plants, FAS showed weak GUS staining, whereas MAS were stained strongly. In the uninfected controls, *pCWLP1*::*GUS* plants had slight GUS staining in the vascular cylinder; however, faint GUS staining was detected in root tips of both *pCWLP1:GUS* and *pBGLU28:GUS* plants (Figure 5).

3.7 | Knocking out host candidate genes alters the sexual fate of nematodes

To investigate which genes/pathways are involved in ESD of nematodes, we selected seven FAS-specific genes (*CWLP1*, *MLP-like protein 423* [*MLP423*], *GLYCEROPHOSPHODIESTER PHOSPHODIESTERASE LIKE 5* [*GDPDL5*], *LACCASE 11* (*LAC11*), *LNG1*, *IRREGULAR XYLEM 12* [*IRX12*], and *GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID PROTEIN TRANSFER 6* [*LPTG6*]) and three MAS-specific genes (*BGLU28*, *BGLU30/DIN2*, and *BASIC HELIX–LOOP–HELIX TRANSCRIPTION FACTOR 101* [*BHLH101*]) for further characterization using loss-of-function T-DNA mutants (Table S1 and Figures S5). Plants were screened for the development of males and females at 2 weeks after inoculation. Out of the eight mutant lines for FAS-specific genes, two lines, *lng1* and *irx12*, showed a significant reduction in the number of females and a significant increase in the number of males as compared with the wild-type Col-0. In addition, the *lptg6* mutant also showed a significant decrease in the number of females as compared with that of Col-0, but the number of males did not change significantly (Figure 6a). We also found that the size of the syncytia, but not the size of the females, was significantly reduced in the lipid transfer protein 6 (*ltpg6*) and *lng1* mutants in comparison with Col-0 plants (Figure 6b,c). By contrast, no changes in nematode numbers were detected in the mutant lines for MAS-specific genes (Figures S6).

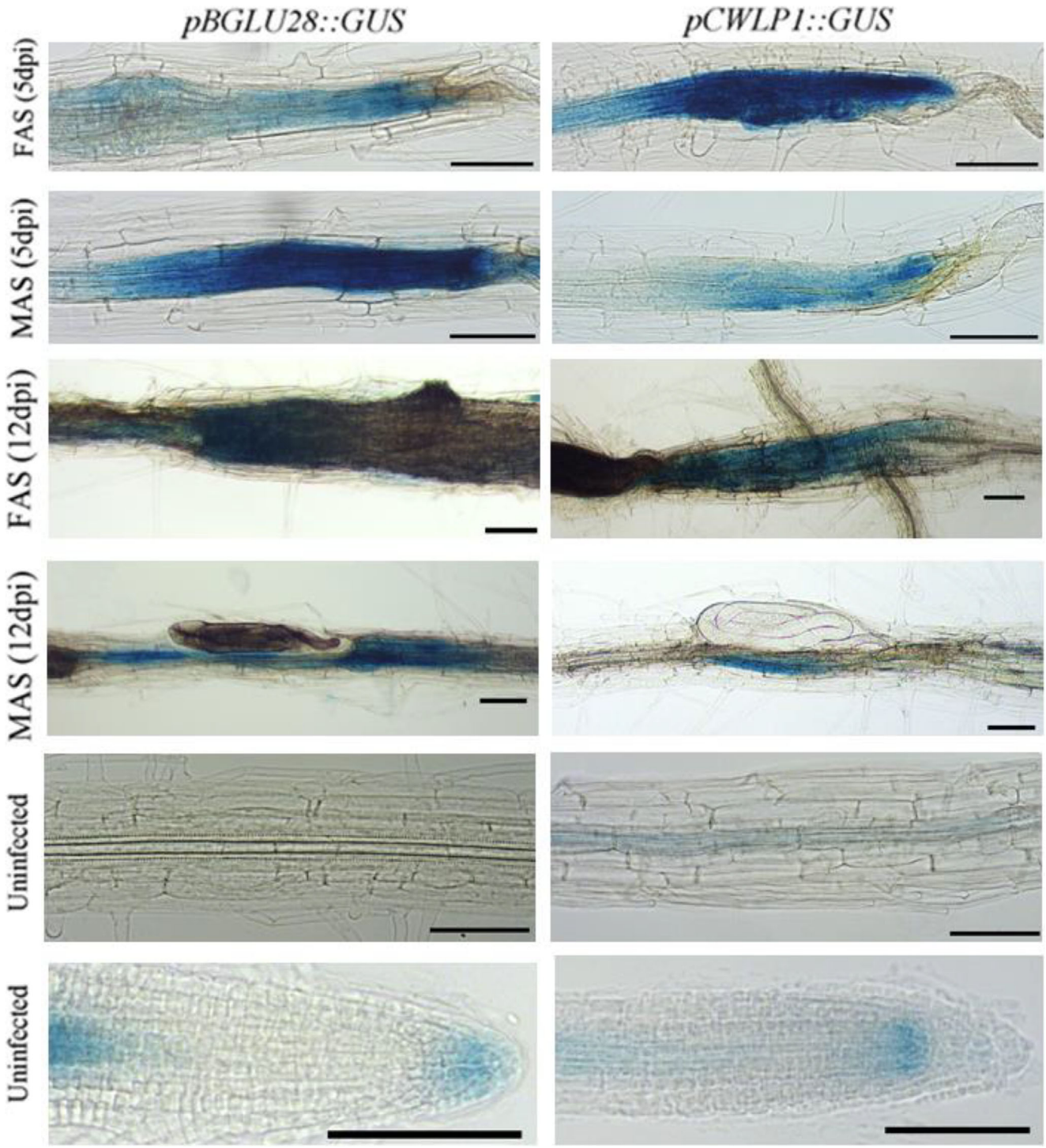

FIGURE 5 β-glucuronidase (GUS) staining of female-associated syncytia and male-associated syncytia at 5 and 12 days postinfection validates microarrays analysis. The figures on the left side represent the pBGLU28::GUS line and on the right side represent the pCWLP1::GUS line of *Arabidopsis*

4 | DISCUSSION

Here, we developed and validated a strategy to predict the sex of cyst nematodes with high certainty during the early stages of infection. The J2 nematodes that developed at the fastest rate during the first 4–5 days after syncytium induction became females, whereas those that grew slower became mainly males. Interestingly, a study by Müller et al. (1981) on comparative food consumption by male and female juveniles from roots of *Brassica napus* found that females consume about 29 times more food than males. Based on our data and previous literature, we concluded that the difference in food consumption leads to the difference in body volume between the sexes.

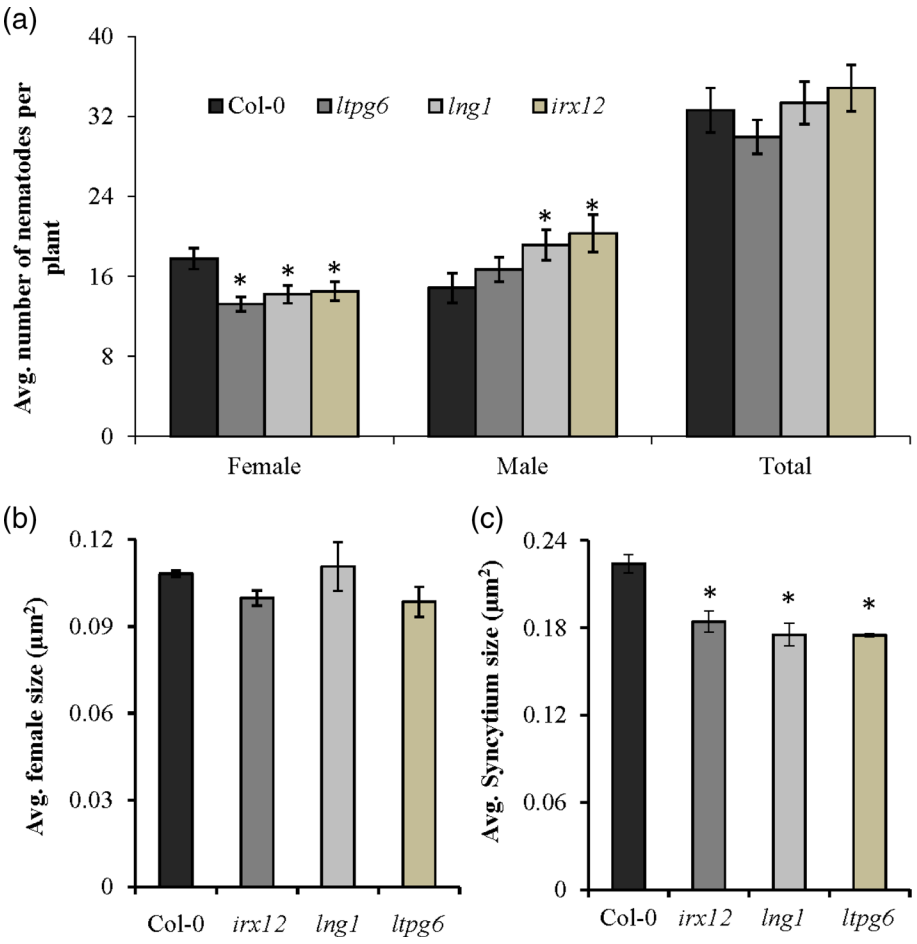

FIGURE 6 Knocking out host candidate genes alters the sexual fate of nematodes. (a) Average number of males, females, and total nematodes. (b) Average syncytium size of FAS. (c) Average female size. (a–c) Bars represent mean ± *SE* from three independent biological replicates ($n = 3$). Data were analysed using two-tailed *t* test. Asterisks represent significant difference from Col-0 (at $p < .05$)

4.1 | Anatomical and ultrastructural differences between FAS and MAS

Microscopic examination showed that FAS and MAS clearly differ in their anatomical organization. MAS were smaller and composed of fewer cells in comparison with FAS. This corresponds to a smaller volume of the syncytium and therefore to a smaller volume of protoplast on which the juveniles feed. Smaller syncytia also have a proportionally reduced surface area of the interface to the vascular cylinder's conductive elements, and thus, the influx of nutrients to MAS may also be reduced. Predicted male juveniles usually migrated inside the vascular cylinder for long distances and caused very extensive cell destruction, which raises the question of whether the continuity of xylem and phloem conductive elements was disrupted. If the conductive system was disrupted, the MAS would be located at its termini, which could affect the access of male nematodes to a continuous flow of nutrients. At the cellular level, the most obvious differences between MAS and FAS was the presence of numerous, small vacuoles in the former and cisternae of rough endoplasmic reticulum in the latter. In general, cisternal rough endoplasmic reticulum is implicated in the biosynthesis of proteins for secretion, whereas small vacuoles, in conjunction with a low electron density of syncytial cytoplasm, might indicate protoplast degradation related to a defence response or programmed cell death. These structural differences indicate that MAS have problems related to nutrient availability or defence responses resembling those observed in resistant plant–nematode interactions (Endo, 1965; Trudgill, 1991).

4.2 | FAS undergoes much intense cell wall modifications as compared with MAS

Earlier transcriptomic studies of syncytia induced in roots of soybean and *Arabidopsis* revealed that nematodes trigger massive changes in key pathways of the host transcriptome including pathways of hormonal regulation, cell wall architecture, cytoskeleton, and dedifferentiation of cells, facilitating the syncytium to function as metabolically highly active cells (Ithal et al., 2007; Puthoff, Nettleton, Rodermel, & Baum, 2003; Szakasits et al., 2009). However, these studies were conducted exclusively on FAS or a mixture of FAS and MAS. We hypothesized that there may be differences in transcriptional regulation between FAS and MAS that in turn influence the sexual differentiation of juveniles. Therefore, we performed a microarray analysis to compare the transcriptomes of MAS and FAS at 5 dpi.

Starting from a single cell, syncytia undergo extensive expansion via dissolution of the cell walls of neighbouring cells. The outer wall of the syncytium is thickened to withstand increased turgor pressure inside the cell (Böckenhoff & Grundler, 1994; Siddique, Sobczak,

Tenhaken, Grundler, & Bohlmann, 2012). Syncytial cell walls in contact with xylem vessels develop numerous ingrowths to enhance the surface area for absorption of nutrients and water (Golinowski et al., 1996; Offler, McCurdy, Patrick, & Talbot, 2003). These changes in cell wall structure help to meet the growing demand for food of developing nematodes. Microarray data published by Puthoff et al. (2003) at 3 dai and Szakasits et al. (2009) at 5 and 15 dpi also showed high upregulation of genes for "cell wall biosynthesis and modifications." Our results indicate that FAS undergo a stronger upregulation of genes for "cell wall biosynthesis," "metabolism," and "modifications" as compared with MAS. This is consistent with the finding that female nematodes require more food and grow much faster in comparison with males (Hofmann et al., 2007; Müller et al., 1981); thus, the upregulation of cell wall-related genes may support the higher nutritional requirement. This hypothesis was also supported by previous studies in which FAS contained more conspicuous cell wall ingrowths (transfer cells) and cell wall openings as compared with MAS (Golinowski et al., 1996; Sobczak, Golinowski, & Grundler, 1997).

4.3 | Immune responses are activated in MAS as compared with FAS

Invasion of the root by nematodes, and subsequent migration towards the vascular cylinder, cause cellular damage and activate plant defence responses (Holbein, Grundler, & Siddique, 2016; Mendy et al., 2017). Nematodes use their stylets to secrete a variety of molecules (effectors) that suppress the defence responses in infected plant cells, leading to the formation of a functional feeding site (Juvale & Baum, 2018). Our transcriptome analysis showed that several plant genes that are activated upon infection by a variety of pathogens are expressed more abundantly in MAS as compared with FAS. Particularly relevant is a set of genes with roles in plant basal defence against pathogens. Among them are members of the polygalacturonase-inhibiting protein gene family (*PGIP1* and *PGIP2*), which are involved in perception and activation of damage-associated defence responses. The expression of both *PGIP1* and *PGIP2* was stronger in MAS than in FAS, and loss-of-function *pgip1* mutants showed a significant increase in the average number of females and a corresponding decrease in the average number of males as compared to Col-0 (Shah et al., 2017). Likewise, several host secondary metabolism genes, such as phytoalexin deficient (*PAD3*), indole glucosinolate o-methyltransferase 1 (*IGMT1*), and cytochrome P450 91A1 (*CYP81D1*), were significantly upregulated in MAS compared with FAS. Intriguingly, we also found that PGIP-mediated changes in host susceptibility to cyst nematodes involve the activation of genes encoding enzymes for host secondary metabolism (Shah et al., 2017).

Our results suggest that ESD in cyst nematodes is influenced by the extent to which host defence responses are avoided/suppressed. We propose that some of the nematodes may be able to effectively suppress or avoid the defence response during the initial stages of infection, leading to the conditions that favour the development of females. In cases where defence responses were established by the plant, the development of males was induced. An alternative theory is that the sex of the beet cyst nematode is genetically predetermined, and male nematodes are not able to effectively suppress the defence responses below a minimum level that is required for the development of females. However, our observations showing that knocking out key defence response genes, such as *PGIP1*, increased the number of females and decreased the number of males makes it unlikely that the sex of the beet cyst nematode is predetermined.

4.4 | MAS shows upregulation of genes involved in nutritional stress response

We found that a number of genes induced by sulphur deficiency, iron deficiency, or starvation were upregulated in MAS as compared with FAS. These observations raise the question of whether the availability of certain essential elements influences the sex differentiation of the nematodes. We propose that nematodes associated with syncytia that are unable to provide them with optimal nutrients may develop as males. This hypothesis is supported by earlier studies suggesting that a deficiency of essential elements, like phosphorous, nitrogen, and potassium, significantly increased the number of males on the host plant (Kämpfe & Kerstan, 1964). Alternatively, it is possible that nematode juveniles provoke a local host defence response during migration and establishment of the ISC, and an inability to effectively suppress or overcome these defence responses may lead to restrictions in the supply of nutrient, leading to the development of males. More work will be needed to investigate this hypothesis.

4.5 | Functional characterization of candidate genes

The candidate genes which were most differentially regulated in both FAS and MAS with respect to each other were selected to observe their possible role in the sex determination of the nematodes. Therefore, infection assays were performed on the *Arabidopsis* T-DNA mutants of these genes. However, in first round, two genes CWLP-1 and BGLU-28, which gave highest differential regulation in FAS and MAS, respectively, did not change any sex ratio in *Arabidopsis* mutants. CWLP-1 is a cell wall linker plasma membrane protein. It is a proline-rich (>30% proline residues) protein where its one domain is anchored in the cell membrane, whereas proline-rich domain protrudes towards cell wall. Proline-rich proteins are known for their role in cell wall development, strengthening, and maintenance (Deepak et al., 2010; Showalter et al., 2010; Stein et al., 2011). During syncytium formation, cell wall undergoes reorganizations and modifications (Böckenhoff & Grundler, 1994; Siddique et al., 2012). Our current transcriptome shows that these changes in the cell wall are more intensive in the case of FAS therefore reflecting highest differentially regulation of CWLP in FAS, whereas no influence on nematode infectivity in T-DNA mutants indicates that CWLP is not a limiting factor in cell wall thickening or modifications during syncytium formation.

The BGLU28 encodes β-glucosidase 28, which is used as sulphur (S) deficiency marker in several studies as it is strongly induced under S-starvation conditions (Dan, Yang & Zheng, 2007; Zhang, 2013). Therefore, it was hypothesized that it releases S by enzymatic breakdown of glucosinolates, plant secondary metabolites (Maruyama-Nakashita et al., 2006; Zheng, Zhang & Leustek, 2014). However, later studies on mutant and overexpressing lines of BGLU28 could not produce any evidences about its role in releasing sulphur for plants under low S environment (Ramamoorthy, 2015). In analysis of our transcriptome, it has been revealed that several genes respond to S-deficiency were differentially expressed in MAS. Therefore, differential regulation of BGLU28 in MAS could be viewed in the context of low sulphur conditions specifically produced in MAS. Moreover, it also has been known that auxin and cytokinin hormones negatively regulate the S-deficiency response including strong down regulation of BGLU28 (Dan, Yang & Zheng, 2007). In FAS, GH3.3, an indole-3-acetic acid amido synthetase, involved in the biosynthesis of auxin was strongly upregulated. This is the possible reason why BGLU28 appeared as highly upregulated in MAS as compared with FAS and no change in sex ratio was observed in T-DNA mutants of BGLU28.

4.5.1 | Irregular xylem 12

Our transcriptome data showed that *IRREGULAR XYLEM 12* (*IRX12*) is one of the differentially expressed genes in FAS compared with MAS. *IRX12* is strongly expressed in vascular bundles and is involved in constitutive lignification of the *Arabidopsis* stem by regulating phenylpropanoid metabolism (Berthet et al., 2011; Brown, Zeef, Ellis, Goodacre, & Turner, 2005). The knockout mutant for *IRX12* exhibits irregular xylem morphology due to the negative pressure produced by water transportation (Brown et al., 2005). The phenotype arises due to defective cellulose and lignin biosynthesis, which would otherwise provide resistance against compressive forces. The phenotype is also an indicator of secondary cell wall malformation (Jones, Ennos, & Turner, 2001; Turner & Somerville, 1997). Our infection assays on the knockout mutant, *irx12*, showed a reduction in the number of females, whereas the number of males was increased significantly compared with the wild type. Because *irx12* mutants are known for defects in lignin biosynthesis and mild reductions in cellulose during secondary cell wall formation of xylem (Hao & Mohnen, 2014; Yokoyama & Nishitani, 2006), we suggest that upregulation of *IRX12* in FAS is required for increased thickening of the syncytial cell wall, which would in turn support an increased tolerance of the syncytia to high turgor pressure. In the absence of *IRX12*, an impairment of cell wall thickening may restrict the enlargement of syncytia, leading to the development of more males.

Alternatively, impairment in secondary cell wall biosynthesis might lead to the activation of plant defence pathways that restrict further development of female nematodes and favour the development of more males. This hypothesis is supported by previous studies where knocking out cell wall biosynthesis genes, such as *IRX5* and *IRX3*, conferred enhanced resistance against necrotrophic and vascular pathogens (Ellis, Karafyllidis, Wasternack, & Turner, 2002; Ramos et al., 2013).

4.5.2 | Glycosylphosphatidylinositol-anchored LTPG6

Nonspecific lipid transfer proteins are plant-specific proteins encoded by large gene family. One of the major types are LTPGs, where the transcript encodes an additional C-terminal signal sequence, which leads to the addition of a glycosylphosphatidylinositol (GPI)-anchor via post-translational modification (Debono et al., 2009). The role of these proteins has been shown to bind and transport lipid molecules in vitro (Carvalho & Gomes, 2007). Therefore, it is assumed that LTPGs are involved in lipid transport to the cell surface in vivo (Edstam & Edqvist, 2014). Several members of LTPGs, including LTPG6, have also been found in phloem exudates of *Arabidopsis*, secreted upon inoculation with *Pseudomonas syringae* pv. tomato. These observations led to the idea that *LTPGs* may be involved in systemic acquired resistance; however, no such role for *LTPGs* has been established (Carella et al., 2016). Nevertheless, knockout mutants for some *LTPGs* in *Arabidopsis* have reduced fertility due to infertile ovules, an inability to restrict the uptake of tetrazolium salt, and decreased levels of ω-hydroxy-hydroxy fatty acids in the seed coat. These studies suggested that *LTPGs* may be involved in the biosynthesis or deposition of suberin or sporopollenin (Edstam & Edqvist, 2014).

Our pathogenicity assays with *ltpg6* mutants showed that although the number of female nematodes was significantly decreased, there was no significant increase in the number of male nematodes. Moreover, the size of FAS was also significantly decreased in the *ltpg6* mutants as compared with that in Col-0. Although, it is hard to identify a role for *LTPG6* in plant–nematode interactions based on current data, *LTPG6* may be involved in suberin biosynthesis and deposition around the feeding site.

4.5.3 | LONGIFOLIA 1

The infection assays on the longifolia 1 (*lng1*) mutant revealed that the number of female nematodes was significantly reduced, and the number of male nematodes was significantly increased. Moreover, the sizes of syncytia were also significantly reduced in the *lng1* mutant as compared with wild-type *Arabidopsis*. The *LNG* gene family consists of only two genes, *LNG1* and *LNG2*, in *Arabidopsis*. Indigenously, they are expressed in various plant organs, including leaves, flowers, and lateral roots where the cellular expression is localized in the cytosol and nucleus. The *LNG1*-overexpression plants have extremely long leaves, elongated floral organs, and elongated siliques. Moreover, *LNG1* and *LNG2* regulate leaf morphology by promoting longitudinal polar cell elongation (Lee et al., 2006). Kerstan (1969) studied the development of males and females in *H. schachtii* with respect to root and giant cell (syncytium) diameter and found that a certain minimum size of syncytium is required for the development of females. Based on our results,

we suggest that in *lng* knockout mutants, the diameter of the syncytium is reduced, which may be less supportive to females, leading to the development of more males. Further studies involving a *lng1 lng2* double mutant may provide more insights into the role of *LNGs* in syncytium and nematode development.

Current transcriptome has shown a variety of genes belonging to various functional categories like defence responses, cell wall organizations, and nutrient deficiency, which were differentially regulated in MAS and FAS. Therefore, targeting the genes according to mentioned categories and performing infection assays on knocked-out and complemented overexpression transgenic lines would reveal the involvement of those genes in manipulating the sex ratio of the nematodes. Thus, the studies will provide important genes that could be used in breeding programs to control nematode's population under field conditions without the use of nematicides.

5 | CONCLUSION

In the present study, we explored the host factors driving the sexual differentiation of the cyst nematode *H. schachtii* at the molecular level. We conclude that a number of factors, including the intensity of the host immune responses, availability of nutrients, and selection of ISC, may contribute to the development of females or males. To our knowledge, this is the first evidence showing that female and male nematodes induce differential gene expression profiles in the same host plant. In the future, it will be important to extend candidate screening to identify additional host mutants with a strong influence on the sex ratio. It will also be critical to further investigate the mechanisms by which these host genes influence the sex of other cyst nematode species. These proposed studies may provide additional resources for the development of nematode-resistant cultivars.

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DATA AVAILABILITY STATEMENT

The paper contains microarrays data, and it is publicly available through array express under the accession number E-MTAB-8648. All other data are within paper or supporting information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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