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WOX11-mediated cell size control in Arabidopsis attenuates growth and fecundity of endoparasitic cyst nematodes

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SUMMARY

Cyst nematodes establish permanent feeding structures called syncytia inside the host root vasculature, disrupting the flow of water and minerals. In response, plants form WOX11-mediated adventitious lateral roots at nematode infection sites. WOX11 adventitious lateral rooting modulates tolerance to nematode infections; however, whether this also benefits nematode parasitism remains unknown. Here, we report on bioassays using a *35S::WOX11-SRDX* transcriptional repressor mutant to investigate whether WOX11 adventitious lateral rooting promotes syncytium development and thereby female growth and fecundity. Moreover, we chemically inhibited cellulose biosynthesis to verify if WOX11 directly modulates cell wall plasticity in syncytia. Finally, we performed histochemical analyses to test if WOX11 mediates syncytial cell wall plasticity via reactive oxygen species (ROS). Repression of WOX11-mediated transcription specifically enhanced the radial expansion of syncytial elements, increasing both syncytium size and female offspring. The enhanced syncytial hypertrophy observed in the *35S::WOX11-SRDX* mutant could be phenocopied by chemical inhibition of cellulose biosynthesis and was associated with elevated levels of ROS at nematode infection sites. We, therefore, conclude that WOX11 restricts radial expansion of nematode-feeding structures and female growth and fecundity, likely by modulating ROS-mediated cell wall plasticity mechanisms. Remarkably, this novel role of WOX11 in plant cell size control is distinct from WOX11 adventitious lateral rooting underlying disease tolerance.

Keywords: cyst nematodes, *Arabidopsis thaliana*, *Heterodera schachtii*, WOX11, cell size, syncytium hypertrophy, female fecundity, cell wall plasticity, ROS.

INTRODUCTION

Biotic stress by endoparasitic cyst nematodes disrupts plant growth by altering the flow of water and minerals through destructive migration within the roots and feeding (Grundler & Hofmann, 2011; Levin et al., 2021; Rodiuc et al., 2014; Trudgill et al., 1975). In response to nematode infection, plants trigger a damage signaling pathway mediated by *WUSCHEL-RELATED HOMEOBOX11* (*WOX11*) that leads to non-canonical root branching, also known as adventitious lateral rooting (Ge et al., 2019; Guarneri et al., 2023). WOX11-mediated adventitious lateral root formation requires the transcription factor LATERAL ORGAN BOUNDARIES DOMAIN16 (LBD16). WOX11 directly binds to the promoter of LBD16, which induces the asymmetric radial expansion of root founder cells (Goh et al., 2012, Vilches Barro et al., 2019). Subsequently, founder cells undergo anticlinal division to initiate an adventitious lateral root primordium. WOX11 adventitious lateral roots compensate for the inhibition of primary root growth caused by cyst nematode infection and contribute to better maintenance of aboveground plant development and growth (Willig et al., 2024). Thus, WOX11 modulates plant tolerance to cyst nematode infections (Willig et al., 2024). Furthermore, WOX11 reduces Arabidopsis susceptibility to cyst nematode penetration (Willig et al., 2024). However, whether WOX11 may affect cyst nematode feeding and thereby female fecundity remains unknown.

Cyst nematode females require a permanent feeding structure to reach the adult stage and produce eggs. Hereto, upon host penetration, the infective second-stage juveniles (J2s) insert their needle-like oral stylet into a cell of the plant vascular cylinder and secrete effector proteins.

As a result, this cell undergoes a cascade of structural changes and fuses with hundreds of neighboring cells by partial cell wall dissolution to form the so-called syncytium. During this process, activation of the endocycle increases the DNA content, while syncytial elements expand radially by hypertrophy (Golinowski et al., 1996). Nematode juveniles feed on syncytia in cycles of continuous ingestion and resting periods, and molt three times until they become adult females and males (Muller et al., 1981). While males stop feeding after the third larval stage and are associated with relatively small syncytia, females ingest food until the adult stage and have large, hypertrophied syncytia (Hofmann & Grundler, 2006; Muller et al., 1981). Syncytial hypertrophy positively correlates with female size, which has been previously used as an indicator of female fecundity (Urwin et al., 1997).

The hypertrophy of female-associated syncytia is thought to depend on the uptake of assimilates, such as sucrose from the phloem (Hofmann & Grundler, 2006). Initially, syncytia are symplastically isolated from surrounding host tissues and sucrose is taken up from phloem companion cells via active transport. Later, when syncytia have reached their maximum expansion, the opening of secondary plasmodesmata allows the passive transport of sucrose from the phloem sieve elements (Hofmann et al., 2007). Increased osmolarity due to the high-sucrose concentration causes the passive inflow of water from the xylem, elevating turgor pressure in the syncytia (Böckenhoff & Grundler, 1994). High turgor pressure poses tensile stress on plant cell walls and is thought to drive syncytial hypertrophy (Cosgrove, 2022; Hofmann & Grundler, 2006). Modifications in the syncytial cell wall composition likely provide mechanical strength to withstand the turgor pressure, while allowing syncytial elements to expand and thus accommodate the periodic demands imposed by nematode feeding (Davies et al., 2012; Zhang et al., 2017). In line with this, silencing of a cell-wall-modifying enzyme involved in cellular hypertrophy compromised female growth in potatoes (Catalá et al., 2000; Karczmarek et al., 2008).

The presence of WOX11-mediated adventitious lateral roots at nematode infection sites might interfere with the flow of assimilates and water toward the syncytium, with possible consequences on syncytium hypertrophy and female fecundity. Indeed, similar to syncytia, adventitious lateral roots constitute a sink of assimilates for the plant (Hofmann et al., 2007; Stitz et al., 2023). As such, adventitious lateral roots may compete with syncytia for the uptake of sucrose from the phloem. Consequently, adventitious lateral root formation may benefit syncytia by enhancing the overall availability of sucrose in the roots. Besides, mature adventitious lateral roots likely increase water flux toward the syncytium (Levin et al., 2020), thereby promoting turgor-driven syncytial hypertrophy. As a result, larger, hypertrophied syncytia may accumulate higher amounts of sucrose, thus better supporting nematode feeding.

Therefore, we hypothesized that WOX11 affects syncytium hypertrophy and thus, female fecundity via the induction of adventitious lateral root formation. We first investigated whether both WOX11 and LBD16 are involved in syncytium hypertrophy, and female growth and fecundity of the beet cyst nematode *Heterodera schachtii* in Arabidopsis. Next, we tested if WOX11-mediated adventitious lateral root formation correlates with both syncytium hypertrophy and female growth. Furthermore, we asked whether WOX11 could directly affect the capacity of syncytia to accommodate large volumes of water and assimilates by modulating plant cell wall plasticity. Finally, given that WOX11 has previously been implicated in the regulation of ROS (Liu et al., 2021; Wang et al., 2021; Xu et al., 2023) and that ROS are known to modulate cell wall plasticity (Eljebbawi et al., 2021), we examined whether WOX11 modulates ROS homeostasis in nematode syncytia. Our findings show that WOX11 attenuates female growth and fecundity, likely by restricting the expansion of syncytial elements. Remarkably, this function of WOX11 in plant cell size control cannot fully be explained by its role in adventitious lateral root formation. Instead, WOX11 may modulate syncytium hypertrophy via ROS-mediated cell wall plasticity mechanisms.

RESULTS

WOX11 restricts the radial expansion of nematode syncytia and attenuates female growth

To test whether WOX11-mediated adventitious rooting may affect nematode parasitism, we first assessed the number of successful infections in the Arabidopsis mutant plants *35S::WOX11-SRDX* (transcriptional repressor) and *lbd16-2* compared to the wild-type Col-0. Hereto, we inoculated 14-day-old plants with infective juveniles of the beet cyst nematode *Heterodera schachtii* and counted the number of females and males per plant at 28 days post-inoculation (dpi). None of the mutants differed from wild-type Col-0 in the number of females or males (Figure 1A,B). Next, we investigated whether WOX11 and LBD16 affect syncytium expansion and female growth. For this purpose, the maximum two-dimensional surface area of the adult females and their corresponding syncytia was measured based on pictures taken at 28 dpi. This showed that the mutant plants *35S::WOX11-SRDX* and *lbd16-2* had significantly larger females and syncytia than wild-type Col-0 (Figure 1C–E). To further understand in which direction (longitudinal or radial) syncytium expansion in the mutants differed from Col-0, we also measured the width and length maxima of syncytia. We found that *35S::WOX11-SRDX* and *lbd16-2* had significantly wider syncytia

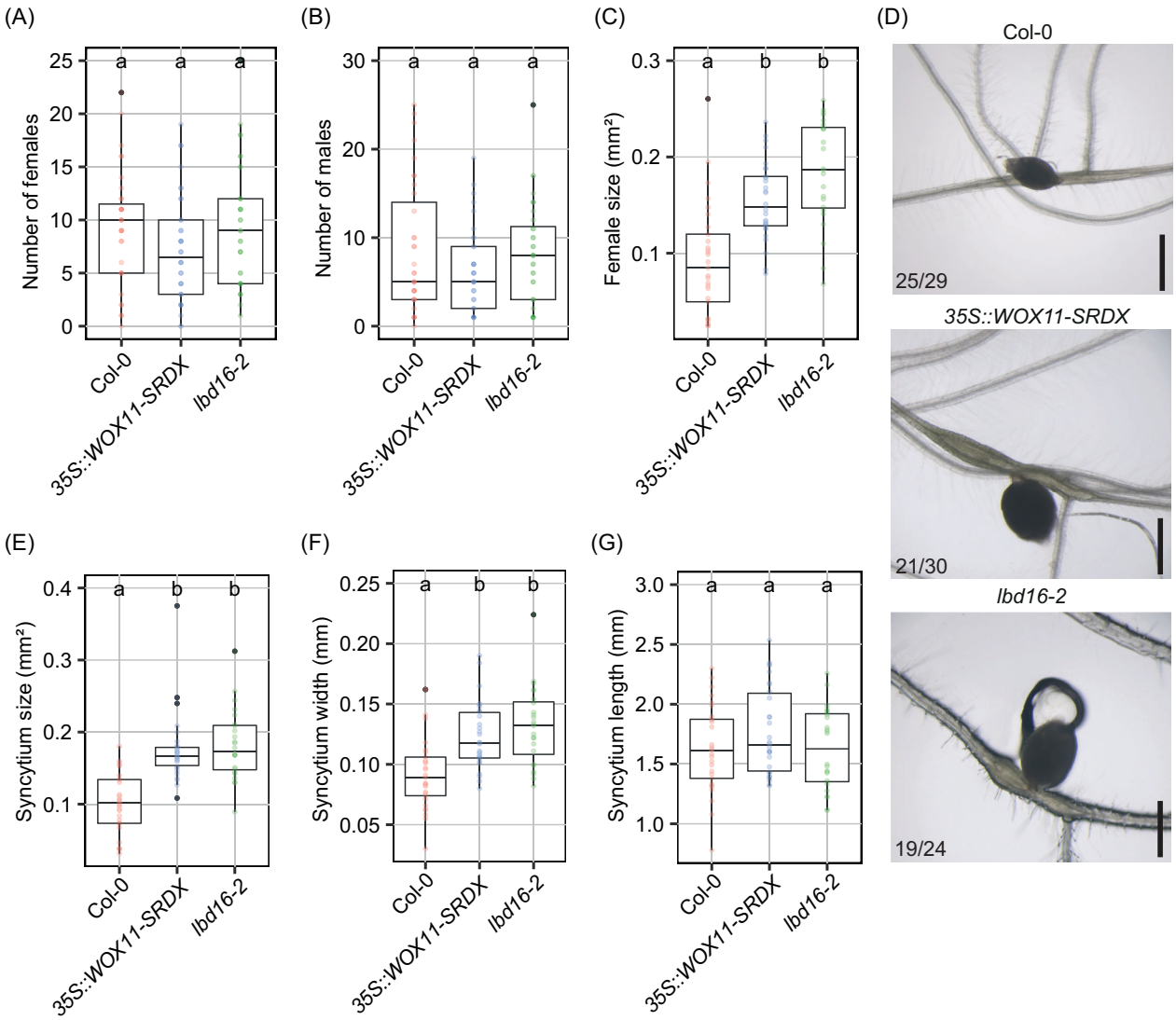

Figure 1. WOX11 and LBD16 restrict *Heterodera schachtii* syncytium hypertrophy and attenuate female growth. Fourteen-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* Arabidopsis plants were inoculated with 250 J2s per plant. Successful infections were assessed at 28 dpi. (A) Number of females. (B) Number of males. (C) Female size. (D) Representative pictures of females and syncytia at 28 dpi. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 0.5 mm. (E) Syncytium size. (F) Syncytium width. (G) Syncytium length. This experiment was performed three times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters indicate statistically different groups ($P < 0.05$, $n = 24$–36).

compared to wild-type plants (Figure 1F). In contrast, no difference in syncytium length was observed among the genotypes (Figure 1G). We concluded that WOX11 and LBD16 restrict the radial expansion of syncytial elements and attenuate female growth.

WOX11 attenuates nematode female fecundity in soil

To assess if WOX11 and LBD16 may also influence female fecundity in soil, we cultivated *35S::WOX11*-SRDX, *lbd16-2*, and wild-type Arabidopsis plants in pots with silver sand and then inoculated them with *H. schachtii*. At the end of the nematode life cycle, the dead and desiccated females, referred to as cysts, were counted and crushed to count the number of eggs or J2s within each cyst (Figure 2). The number of cysts from *35S::WOX11-SRDX* plants was not significantly different from the wild-type Col-0. However, *lbd16-2* plants had a lower number of cysts per plant compared to Col-0, suggesting that LBD16 may enhance plant susceptibility to nematode infections in pot conditions (Figure 2A). Interestingly, we found that the average number of eggs or J2s per cyst was significantly higher in *35S::WOX11-SRDX* compared to Col-0 plant. Although *lbd16-2* also showed a higher average number of eggs or J2s per cyst than Col-0, this difference was not significant

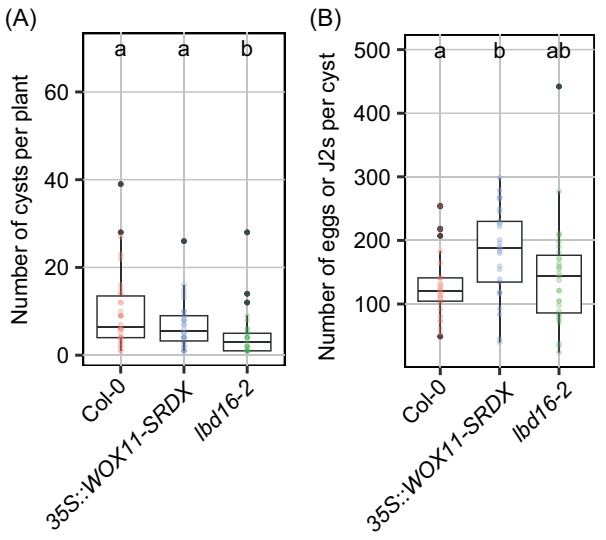

Figure 2. WOX11 attenuates *Heterodera schachtii* female fecundity in soil. Twenty-one days after growing wild-type Col-0 and *35S::WOX11-SRDX* and *lbd16-2* mutants on silver sand, plants were inoculated with 25 J2s/g of dry sand. At 28 dpi, cysts were extracted, counted, and crushed to count the number of eggs or J2s contained in each cyst. (A) Number of cysts per plant. (B) Average number of eggs or J2s per cyst. Significance of differences between genotypes was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters indicate statistically different groups ($P < 0.05$, $n = 30–35$).

(Figure 2B). Thus, we concluded that WOX11 does not affect the number of females developing into cysts but restricts female fecundity in soil.

The role of WOX11 on syncytium hypertrophy and female growth is distinct from its function in adventitious lateral root formation

Given the negative effect of WOX11 on *H. schachtii* female growth and fecundity, we questioned whether WOX11-mediated adventitious lateral root formation at nematode syncytia may interfere with nematode feeding. Indeed, adventitious lateral roots are strong sinks of assimilates (Stitz et al., 2023), and therefore could compete with syncytium hypertrophy and female growth. To test this, we performed a correlation analysis between the number of adventitious lateral roots emerging from syncytia and both syncytium and female size in Col-0, *35S::WOX11*-SRDX, and *lbd16-2*. In contrast to what we hypothesized, we found a moderately positive correlation between the number of adventitious lateral roots emerging from syncytia and both syncytium and female size in wild-type Col-0 (Figure 3A,B). Thus, the emergence of adventitious lateral roots may support syncytium hypertrophy and female growth. Moreover, this positive correlation was not observed in the *35S::WOX11*-SRDX and *lbd16-2* mutants (Figure 3A,B). This indicates the role of WOX11 in syncytium hypertrophy and female growth is distinct from its function in adventitious lateral root formation.

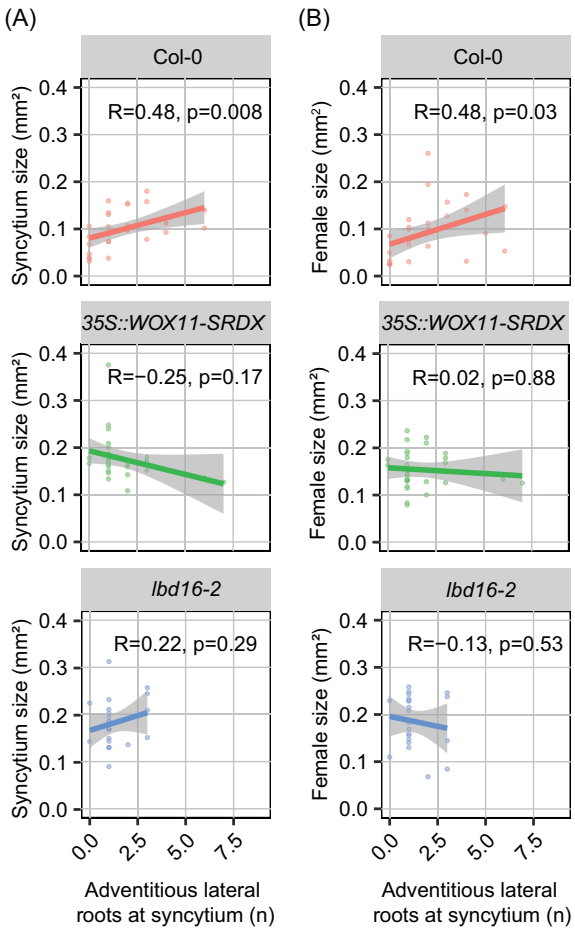

Figure 3. The effect of WOX11 and LBD16 on *Heterodera schachtii* syncytium hypertrophy and female growth cannot fully be explained by their role in adventitious lateral root formation. Fourteen-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* plants were inoculated with 250 *H. schachtii* J2s per plant. At 28 dpi, the number of adventitious lateral roots in contact with nematode syncytia was counted and the size of females and syncytia was measured. (A) Correlation between the number of adventitious lateral roots and syncytium size. (B) Correlation between the number of adventitious lateral roots and female size. This experiment was performed three times with similar outcomes and data were pooled for statistical analysis. Correlation (R) between two variables was calculated using Spearman's rank-order correlation coefficient ($n = 24–30$). Gray area indicates 95% confidence interval.

WOX11 restricts the early radial expansion of syncytial elements

Radial expansion of nematode syncytia is mainly determined by the cellular hypertrophy of syncytial elements at 5 dpi (Golinowski et al., 1996; Magnusson & Golinowski, 1991). At this stage, syncytial elements are still symplastically isolated from the surrounding host tissue, which likely enables the buildup of turgor pressure required for cellular hypertrophy (Hofmann et al., 2007; Ruan et al., 2004). Since we previously observed that WOX11 and LBD16 are expressed between 2 and 7 dpi (Willig et al., 2024), we asked whether WOX11 and LBD16 play a role in

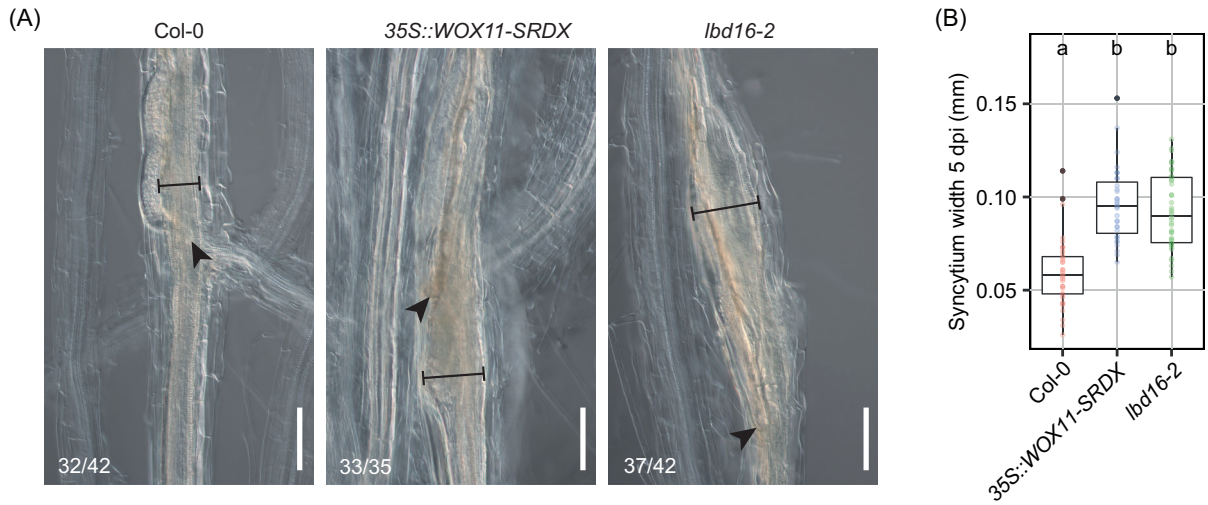

Figure 4. WOX11 restricts the early radial expansion of *Heterodera schachtii* syncytial elements. Four-day-old Arabidopsis seedlings were inoculated with 15 *H. schachtii* J2s per plant. At 5 dpi, pictures were taken of syncytia, and width was measured. (A) Representative pictures of syncytia at 5 dpi. Black arrowheads indicate the nematode head. Black lines indicate syncytium width. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 100 μm. (B) Syncytium width at 5 dpi. This experiment was performed three times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters indicate statistically different groups ($P < 0.001$, $n = 35–42$).

the early phases of syncytium expansion. To this end, we measured syncytium width in *35S::WOX11-SRDX* and *lbd16-2* mutants at 5 dpi. We found that already at such an early time point, the two mutants displayed wider syncytia compared to the wild-type Col-0 (Figure 4A,B). Thus, WOX11 and LBD16 limit the early radial expansion of syncytial elements.

WOX11 restricts the cell wall extensibility of syncytial elements

Root cells in the maturation zone are typically long and narrow. This is because root cells elongate longitudinally in the root elongation zone before completing differentiation and entering the maturation zone (Verbelen et al., 2006). The orientation of cellulose microfibrils and the properties of other cell wall components increase the stiffness of the lateral cell walls, thus guiding the expansion in the longitudinal direction (Chaudhary et al., 2020). However, plant developmental processes and environmental stresses can cause changes in cell wall structure and composition, leading to enhanced radial expansion of root cells (Gigli-Bisceglia et al., 2020). It was previously shown that LBD16 regulates the asymmetric radial expansion of founder cells, which involves the differential organization of cortical microtubules driving the deposition of cellulose microfibrils (Vilches Barro et al., 2019). Here, we hypothesized that WOX11 and LBD16 restrict the radial expansion of syncytial elements by modulating cell wall plasticity mechanisms.

To test this, we inhibited cellulose biosynthesis using isoxaben, a chemical that causes the internalization of cellulose synthase complexes from the plasma membrane to cytosolic vesicles (Tateno et al., 2016). Disruption of cellulose biosynthesis or changes in cellulose microfibril alignment affects the directional growth of plant cells, which causes the cells to become radially swollen. Hence, 4-day-old Arabidopsis *35S::WOX11-SRDX*, *lbd16-2*, and wild-type Col-0 seedlings were inoculated with *H. schachtii*. At 5 dpi, seedlings were treated either with isoxaben or DMSO as a negative control. Isoxaben treatment led to a significant increase in syncytium width in wild-type Col-0 plants, which phenocopied the DMSO-treated syncytia in the *35S::WOX11-SRDX* and *lbd16-2* mutants (Figure 5A,B). Thus, inhibition of cellulose biosynthesis causes similar effects on radial expansion of syncytial elements as a disruption in WOX11- and LBD16-mediated pathways.

Interestingly, isoxaben treatment did not have a visible additive effect on the radial expansion of syncytia in *35S::WOX11-SRDX* and *lbd16-2* (Figure 5A,B), suggesting that the syncytial cell walls in the mutants already reached their maximum extensibility. Therefore, we hypothesized that WOX11 and LBD16 attenuate plant cell wall extensibility. We verified this by treating 9-day-old non-infected seedlings with isoxaben, which is known to cause the radial expansion of root epidermal cells at the elongation zone (Chaudhary et al., 2020). After 5 hours of treatment with isoxaben, we found that the width of epidermal cells at the elongation zone of *35S::WOX11-SRDX* and *lbd16-2* increased dramatically compared to wild-type Col-0 (Figure 6A,B). Notably, the width of epidermal cells in all genotypes was similar upon treatment with the negative control (Figure 6A,B). Altogether, our data suggest that

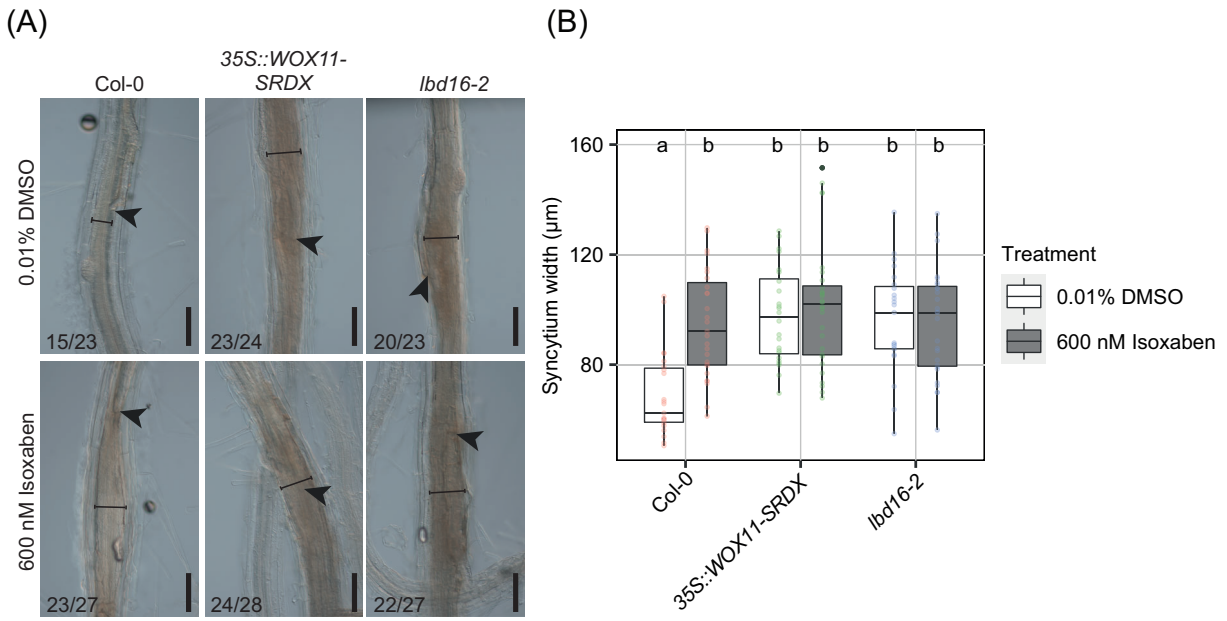

Figure 5. Chemical inhibition of cellulose biosynthesis in Col-0 phenocopies the enhanced radial expansion of syncytial elements observed in *35S::WOX11-SRDX* and *lbd16-2*. Four-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* Arabidopsis seedlings were inoculated with 15 *H. schachtii* J2s. At 5 dpi, seedlings were treated with either 600 nM isoxaben or a mock solution (0.01% DMSO). (A) Representative pictures of nematode syncytia. Black arrowheads indicate the nematode head. Black lines indicate syncytium width. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 100 µm. (B) Quantification of syncytium width. This experiment was performed two times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons ($P < 0.05$, $n = 23–27$). Different letters indicate statistically different groups.

WOX11 and LBD16 restrict cell wall extensibility of both isoxaben-treated epidermal cells at the elongation zone and syncytial elements in the mature zone.

WOX11 modulates ROS homeostasis in nematode syncytia

ROS homeostasis is important in determining plant cell wall plasticity (Eljebbawi et al., 2021). WOX11 was previously found to regulate ROS homeostasis for crown root development in rice (Xu et al., 2023) and during drought and salt stress in poplar (Liu et al., 2021; Wang et al., 2021). Therefore, we hypothesized that WOX11-mediated plant cell wall plasticity involves the regulation of ROS. First, we stained 5 dpi syncytia in the *35S::WOX11-SRDX* and *lbd16-2* mutants with a DAB solution, which is oxidized by peroxidases in the presence of H_2O_2 (Eljebbawi et al., 2021). This revealed that nematode-infected roots of the *35S::WOX11-SRDX* and *lbd16-2* mutants have significantly lower ROS compared to wild-type Col-0 (Figure 7A, B). Thus, we concluded that WOX11 modulates ROS homeostasis upon nematode infection.

To verify whether WOX11-mediated ROS burst is linked to cell wall plasticity responses, we treated non-infected 9-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* seedlings with 600 nM isoxaben or 0.01% DMSO for 5 h, followed by DAB staining. Isoxaben treatment caused an increase in ROS at the elongation zone in all genotypes. However, the levels of ROS in the *35S::WOX11-SRDX* and *lbd16-2* mutants were significantly lower compared to Col-0 (Figure 8A,B). Thus, we concluded that modulation of ROS homeostasis is a plausible mechanism underlying WOX11-mediated cell wall plasticity at nematode syncytia.

DISCUSSION

Plant developmental plasticity mitigates the negative impacts of cyst nematode infections on growth, yet its potential impact on nematode parasitism remains largely unknown. We recently reported that plant perception of cyst nematode invasion induces the formation of WOX11 adventitious lateral roots at nematode infection sites (Guarneri et al., 2023; Willig et al., 2024). Through this local root plasticity response, WOX11 compensates for the inhibition of primary root growth caused by nematode infection, which benefits overall plant growth and development (Willig et al., 2024). In this study, we shift perspective from the plant to the nematode and investigate whether WOX11-mediated developmental plasticity has an impact on nematode growth and fecundity. Our findings support a model where WOX11 modulates ROS homeostasis and cell wall plasticity mechanisms that attenuate syncytial cell size and nematode fecundity.

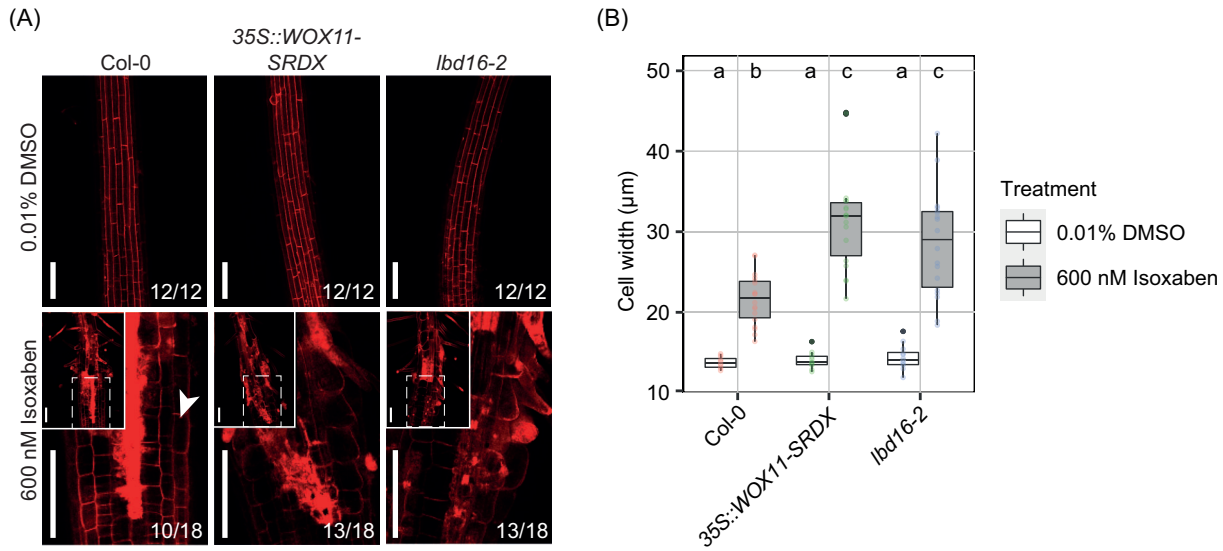

Figure 6. WOX11 restricts the radial expansion of epidermal cells at the elongation zone in response to isoxaben treatment. Nine-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* Arabidopsis seedlings were treated with either 600 nM isoxaben or a mock solution (0.01% DMSO) and then mounted in propidium iodide for imaging. (A) Representative pictures of the elongation zone. White arrowheads indicate a radially expanded epidermal cell. The top-left inserts represent the original zoomed-out pictures. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 100 μm. (B) Quantification of epidermal cell width. This experiment was performed two times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons ($P < 0.0001$, $n = 12–18$). Different letters indicate statistically different groups.

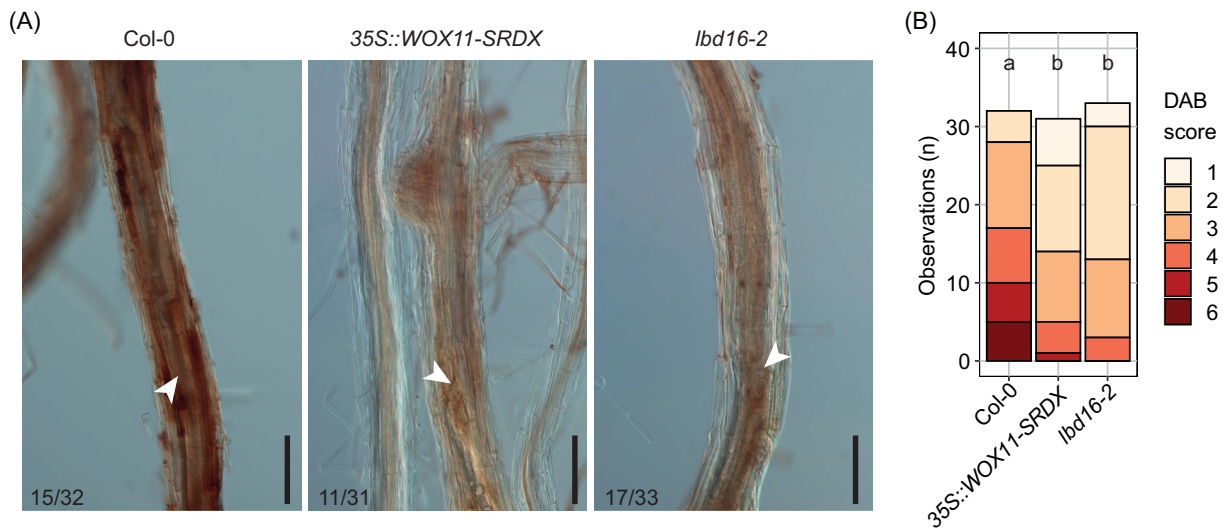

Figure 7. WOX11 and LBD16 increase ROS levels in *Heterodera schachtii*-infected *roots. Four-day-old Col-0,* 35S::WOX11-SRDX, *and* lbd16-2 *Arabidopsis seedlings were inoculated with 15* H. schachtii *J2s. At 5 dpi, seedlings were incubated in a DAB staining solution. (A) Representative pictures of nematode syncytia. White arrowheads indicate the nematode head. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 100 μm. (B) Scoring of DAB staining intensity on a scale from 1 to 6 based on the pictures shown in Figure S2. Bar graphs indicate how often a certain DAB score is observed in each genotype. This experiment was performed three times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by aligned ranks transform (ART) non-parametric ANOVA followed by an ART contrast test for multiple comparisons (P < 0.0001,* n = *31–35). Different letters indicate statistically different groups.*

We provide evidence that WOX11 restricts cyst nematode female growth and egg production. Although it was previously found that WOX11 decreases host invasion by infective juveniles (Willig et al., 2024), we observed that the repressor *35S::WOX11-SRDX* did not alter the number of nematodes establishing a permanent infection. Yet, Arabidopsis roots of the *35S::WOX11-SRDX* repressor displayed bigger females compared to wild-type Col-0 *in*

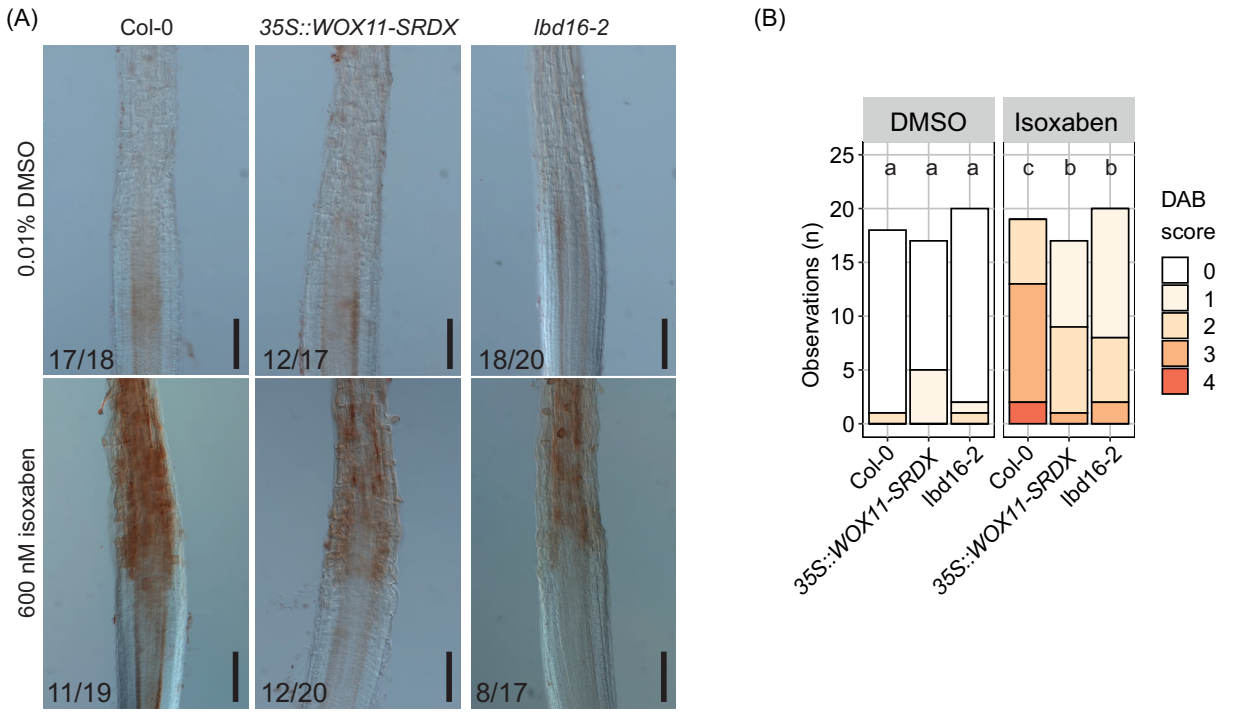

Figure 8. WOX11 and LBD16 increase ROS levels at the elongation zone in response to cellulose biosynthesis inhibition. Nine-day-old Col-0, *35S::WOX11-SRDX*, and *lbd16-2* Arabidopsis seedlings were transferred to liquid KNOP medium containing either 600 nM isoxaben or 0.01% DMSO. After 5 h, seedlings were incubated in a DAB staining. (A) Representative pictures of the elongation zone. Numbers at the bottom left corner indicate how often a similar phenotype as shown in the representative pictures was observed. Scale bar is 100 μm. (B) Scoring of DAB staining intensity on a scale from 0 to 4, based on the pictures shown in Figure S2. Bar graphs indicate how often a certain DAB score is observed in each genotype. This experiment was performed two times with similar outcomes and data were pooled for statistical analysis. Significance of differences between genotypes was calculated by aligned ranks transform (ART) non-parametric ANOVA followed by an ART contrast test for multiple comparisons ($P < 0.0001$, $n = 18–20$). Different letters indicate statistically different groups.

vitro. When plants cultivated in soil were inoculated with *H. schachtii*, the cysts extracted from *35S::WOX11-SRDX* contained a higher number of eggs and J2s as compared to the cysts obtained from wild-type Col-0.

Consistently, the downstream target of WOX11, LBD16, similarly decreased female growth. In fact, *lbd16-2* plants grown *in vitro* showed bigger females compared to wild-type Col-0 plants. In soil, the mutant displayed a higher number of eggs or J2s per cyst compared to Col-0, albeit this difference was not statistically significant. It is important to note here that the outcomes of nematode bioassays in soil are generally more variable and have a lower resolution than *in vitro* assays, as plant growth in soil is subjected to additional stresses and more variable conditions (Grenier et al., 2020). LBD16 had no effect on the number of nematodes that successfully established an infection *in vitro*. This is in agreement with a previous study, where the repressor mutant *35S::LBD16-SRDX* did not alter the number of nematode-induced syncytia after inoculation with *H. schachtii* (Cabrera et al., 2014). However, the number of cysts extracted from *lbd16-2* plants grown in soil was lower compared to Col-0 and *35S::WOX11-SRDX*. This may be due to LBD16 not only regulating WOX11-dependent adventitious rooting but also WOX11-independent lateral root formation (Sheng et al., 2017). Thus, the disruption of both rooting pathways in the *lbd16-2* may have compromised nematode parasitism in soil, negatively impacting the development of cysts and their egg content.

In contrast with our initial hypothesis, we found that WOX11-mediated female growth cannot fully be explained by the role of WOX11 in adventitious lateral root formation. Our data showed that adventitious lateral roots positively correlate with syncytium hypertrophy and female growth in wild-type plants. This may be due to adventitious lateral roots increasing water influx toward nematode syncytia, as previously discussed by Levin et al. (2020). Nevertheless, the positive correlation between the number of adventitious lateral roots and syncytium and female size was disrupted in the repressor *35S::WOX11-SRDX* and the *lbd16-2* mutants. This suggested that in addition to adventitious lateral root formation, WOX11 modulates syncytium hypertrophy and nematode feeding.

WOX11 may attenuate female offspring size by directly restricting the availability of plant assimilates in nematode syncytia. We found that the repressor *35S::WOX11-SRDX* and *lbd16-2* enhanced the radial expansion of syncytia at 5 dpi, a stage where syncytial elements are

symplastically isolated from the surrounding host tissue (Hofmann et al., 2007). During symplastic isolation, nematode syncytia take up sucrose from the phloem via the apoplast through active transporters, which attracts water from the xylem and builds up turgor pressure (Grundler & Hofmann, 2011; Hofmann et al., 2007). The cyclic feeding behavior of nematodes likely causes fluctuations in sucrose levels and turgor pressure that are accommodated by changes in cell wall plasticity and syncytium expansion (Zhang et al., 2017). If WOX11 restricts the radial expansion of syncytial elements, it could interfere with the ability of syncytia to accumulate assimilate, and support the cyclic food demands of female nematodes.

Furthermore, our data suggest that WOX11 modulates the plasticity of syncytial cell walls. We found that inhibition of cellulose biosynthesis using isoxaben increased the radial expansion of syncytial elements similar to the disruption of WOX11-mediated pathways. Moreover, in non-infected plants, WOX11 limited the radial expansion of epidermal cells at the elongation zone in response to isoxaben treatment. This indicates that WOX11 limits the extensibility of syncytial elements in the radial direction. Syncytial cell wall plasticity plays an important role in cyst nematode parasitism. Indeed, the silencing of two plant cell-wall-modifying endo-β-1,4-glucanases reduced the number of females and their egg content in potatoes (Karczmarek et al., 2008). Endo-β-1,4-glucanases loosen the cell wall by modifying amorphous cellulose structures (Glass et al., 2015), which likely affects the expansion of syncytial elements. Moreover, the upregulation of many expansin proteins in nematode syncytia at 5 dpi suggests that syncytium expansion involves mechanisms of cell wall loosening (Wieczorek et al., 2006). It is possible that WOX11 mediates cell wall plasticity in nematode syncytia by regulating the expression of expansin genes. Indeed, WOX11 was found to directly bind the promoter of an expansin gene and thereby modulate grain width in rice (Xiong et al., 2023).

Plant cell wall plasticity is a tightly regulated process involving many interacting components (Cosgrove, 2022). Organization and biosynthesis of cellulose microfibrils, the major load-bearing components of the cell wall, are strongly influenced by cortical microtubules and by the properties and abundance of different polymers in the cell wall matrix (Du et al., 2020; Li et al., 2015; Xiao et al.,2015). Cortical microtubules guide cellulose synthase complexes, determining the organization of cellulose microfibrils (Li et al., 2015). In turn, cellulose deposition regulates microtubule organization through a positive feedback loop (Vilches Barro et al., 2019). Moreover, pectin methylation and hemicellulose were found to affect both cellulose biosynthesis and cortical microtubule organization (Du et al., 2020, Xiao et al., 2015). Due to this complex network, isoxaben treatment not only causes internalization of cellulose synthase complex but also alters the organization of cortical microtubules (Vilches Barro et al., 2019). We observed that isoxaben treatment did not result in a measurable radial expansion of nematode syncytia in the *35S::WOX11-SRDX* and *lbd16-2* mutants. Interestingly, syncytium expansion is known to involve the disorganization of cortical microtubules (De Almeida Engler et al., 2004). Thus, one plausible explanation for our observation is that WOX11 and LBD16 mediate plant cell wall plasticity mechanisms that alter the organization of cortical microtubules. In this scenario, the level of microtubule organization in *35S::WOX11-SRDX* and *lbd16-2* syncytia may be too low to be further affected by isoxaben. Consistently, cortical microtubule organization was found to play an important role in LBD16-mediated asymmetric radial expansion of root founder cells (Vilches Barro et al., 2019).

Our study suggests that WOX11 modulates ROS homeostasis in nematode syncytia. We found that WOX11 and LBD16 increase ROS accumulation in 5 dpi nematode syncytia. This is in line with a recent study where WOX11 directly activates peroxidase activity to induce ROS production and regulate crown root development in rice (Xu et al., 2023). Besides, WOX11 was found to reduce cytotoxic levels of ROS in response to salt and drought stress in poplar (Liu et al., 2014; Wang et al., 2021). Thus, WOX11 modulates ROS homeostasis in different plant species and in response to multiple stresses. However, how WOX11-mediated ROS homeostasis affects plant developmental plasticity still remains unclear.

WOX11-mediated ROS homeostasis could modulate the extensibility of plant cell walls. ROS regulate both cell wall loosening and cell wall stiffening (Eljebbawi et al., 2021; Schmidt et al., 2016). H_2O_2 can be converted by peroxidases into OH^-, which catalyzes the oxidative cleavage of hemicellulose and pectins in the apoplast and loosens the cell wall (Eljebbawi et al., 2021). Additionally, when H_2O_2 levels are high, peroxidases promote the oxidation and thereby cross-linking of extensins and phenolic compounds, leading to cell wall stiffness (Brownleader et al., 2000; Eljebbawi et al., 2021; Magliano & Casal, 1998). Furthermore, ROS homeostasis regulates tubulin polymerization during microtubule formation (Livanos et al., 2012), which could affect the orientation of cellulose biosynthesis and hereby change the cell wall structural properties (Li et al., 2015). We have observed that WOX11 induces ROS production at the elongation zone in response to cellulose biosynthesis inhibition by isoxaben. Moreover, WOX11 attenuates the radial expansion of epidermal cells at the elongation zone upon isoxaben treatment. Thus, we suggest that WOX11-mediated ROS homeostasis increases cell wall stiffness in response to cellulose biosynthesis inhibition, thereby preventing further swelling of cells at the elongation zone. Similarly, WOX11 could modulate ROS to restrict syncytial cell wall extensibility. Whether

this involves the regulation of cortical microtubules, the cross-linking of extensins and phenolics, or other cell wall plasticity mechanisms needs to be investigated.

Besides regulating root development, ROS are key players in plant immunity (Wu et al., 2023). It is therefore possible that lower ROS levels in the *35S::WOX11-SRDX* mutant weakened plant immunity, allowing for a higher nematode penetration (Willig et al., 2024) and increased female performance. Yet, the number of nematodes that developed into adults remained unchanged compared to wild-type plants, suggesting that the higher penetration rate in the mutant might have been counterbalanced by a lower syncytium establishment rate. Previous research showed that ROS inhibit cell death and promote syncytium initiation (Chopra et al., 2021; Siddique et al., 2014). Thus, WOX11-mediated ROS accumulation might facilitate syncytium initiation, although this does not affect the endpoint number of adult nematodes infecting plant roots.

In conclusion, we showed that WOX11 controls cell size likely by modulating ROS homeostasis and cell wall plasticity. Furthermore, we demonstrated that WOX11 attenuates female growth and offspring size. Our results point to a novel role of WOX11 in the plant response to nematode infection, which is distinct from WOX11-mediated adventitious rooting. Importantly, our findings provide evidence that plant developmental plasticity can modulate nematode parasitism.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The Arabidopsis (*Arabidopsis thaliana*) lines Col-0, *35S::WOX11-SRDX* (Liu et al., 2014), and *lbd16-2* (Fan et al., 2012) were used. The transcriptional repressor *35S::WOX11-SRDX* mutant was chosen since it was reported to be more strongly impaired in adventitious lateral root formation compared to the double-mutant *wox11-2 wox12-1* (Liu et al., 2014; Sheng et al., 2017). For *in vitro* experiments, Arabidopsis seeds were vapor sterilized and grown on modified Knop medium (Sijmons et al., 1991) in a growth chamber with a 16 h: 8 h, light: dark photoperiod at 21°C. Plants were grown horizontally in 12-well plates (one plant per well) for the *in vitro* infection assay and vertically in 12 × 12 cm Petri dishes (10 seedlings per plate) for microscopy experiments. For pot experiments, non-sterile Arabidopsis seeds were sown on top of silver sand and grown under greenhouse conditions with 19–21°C and a 16 h: 8 h, light: dark photoperiod. Plants were watered regularly with a Hyponex solution as previously described in Willig et al. (2023).

Nematode sterilization and *in vitro* inoculation

Heterodera schachtii (Woensdrecht population from IRS, The Netherlands) cysts were obtained from infected *Brassica oleracea* roots grown in sand (Baum et al., 2000). The cysts were hatched for 7 days in a solution containing 1.5 mg ml^{-1} gentamycin sulfate, 0.05 mg ml^{-1} nystatin, and 3 mM $ZnCl_2$. Next, *H. schachtii* second-stage juveniles (J2s) were separated from debris using a 35% sucrose gradient and incubated in a sterilization solution (0.16 mM $HgCl_2$, 0.49 mM NaN_3, and 0.002% Triton X-100) for 15 minutes. Finally, the J2s were washed three times with sterile tap water and re-suspended in 0.7% Gelrite (Duchefa Biochemie, Haarlem, The Netherlands). Ten microliter of gelrite solution containing 250 J2s was inoculated onto the roots of 14-day-old Arabidopsis plants for the *in vitro* infection assay, whereas 5 μl drops containing 15 J2s were pipetted onto the younger 4-day-old seedlings used for microscopy experiments.

Pot experiment

Twenty-one-day-old Arabidopsis plants were inoculated with 25 non-sterile J2s per gram of dry sand. This inoculation density was previously found to yield enough (approximately 10) cysts without causing excessive inhibition of plant growth (Willig et al., 2023; Figure S1). Distribution of the pots containing the different genotypes in the trays followed a randomized block design. At 28 days post-inoculation (dpi), watering of the plants was discontinued and sand within the pots was allowed to dry for 1 month. The shoots were cut off and each pot was wrapped in aluminum foil and autoclaved. Samples were sent to the NAK (Emmeloord, The Netherlands) for automated cyst extraction. Cysts were crushed and the number of eggs and J2s per cyst was counted following the protocol by Teklu et al. (2018).

Isoxaben treatment

The procedure for the isoxaben treatment was adapted from literature (Chaudhary et al., 2020). Four-day-old Arabidopsis seedlings were inoculated with 15 J2s per seedling or with a mock solution. At 5 dpi, seedlings were transferred to 55 mm round petri dishes containing 10 ml of liquid Knop medium and either 600 nM isoxaben or 0.01% DMSO. After 5 h of treatment, seedlings were transferred to fresh liquid Knop medium. Nematode syncytia were imaged by brightfield microscopy as described in the next section. Roots were mounted in 10 μg ml^{-1} propidium iodide staining to image the elongation zone of non-infected seedlings and pictures were taken using a Leica SP8 confocal microscope (-excitation/emission 488/600–640 nm). For each sample, the one-dimension size (maximum width) of five epidermal cells at the elongation zone was measured using Fiji software (Schindelin et al., 2012), after which the average width was calculated.

Measurement of female and syncytium size

For the observation of syncytia at 5 dpi, seedlings were mounted in water and imaged with an Axio Imager.M2 light microscope (Zeiss) via a × 20 objective and a differential interference contrast filter. To image mature females and syncytia at 28 dpi, an Olympus SZX10 binocular (Olympus, Tokyo, Japan) with a × 1.5 objective and ×2.5 magnification was used. Pictures were taken with an AxioCam MRc5 camera (Zeiss). Infection sites with only one nematode per syncytium were selected. The size of females and syncytia was extracted from the pictures by manually measuring the maximum two-dimensional surface areas using Fiji software (Schindelin et al., 2012). The length of syncytia was taken by drawing a longitudinal line in the middle of the syncytium. The width was measured by selecting the widest point of the syncytium.

DAB staining

Five days after inoculation, infected and non-infected Arabidopsis seedlings were stained with DAB as described previously (Siddique et al., 2014). First, seedlings were incubated in a DAB staining solution (10 mg ml^{-1} in water) for 2 hours in the dark. Then, they were bleached using an ethanol: lactic acid: glycerol (3:1:1) solution.

Finally, seedlings were mounted in water and imaged with an Axio Imager.M2 light microscope (Zeiss) via a × 20 objective and a differential interference contrast filter. DAB staining intensity was scored on a scale from 0 to 6 as described in Figure S2.

Statistical analyses

Data were analyzed using the R software version 3.6.3 (Windows, x64). The R packages used were tidyverse (https://CRAN.R-project.org/package=tidyverse), ARTool (https://CRAN.R-project.org/package=ARTool), and multcompView (https://CRAN.R-project.org/package=multcompView). Correlation between variables was calculated using Spearman rank-order correlation coefficient. The 95% confidence interval of the linear models was calculated using geom_smooth in R. For normally distributed data, significance of the differences among means was calculated by ANOVA followed by Tukey's HSD test for multiple comparisons. For non-parametric two-factorial ANOVA, an aligned rank transform followed by an ART contrast test for multiple comparisons was performed.

AUTHOR CONTRIBUTIONS

GS, NG, and JJW conceived the project. NG and JJW designed the experiments and performed data collection. Data were analyzed and interpreted by NG, GS, JJW, JJLT, MGS, and PN. NG and GS wrote the article with inputs from AG, JJLT, JJW, MGS, PN, and VW.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supplementary Material of this article.

SUPPORTING INFORMATION

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

Figure S1. Number of *Heterodera schachtii* cysts at increasing inoculation densities in wild-type Col-0 grown in pots.

Figure S2. Scale used for scoring the DAB staining of Arabidopsis roots.

Figure S3. DAB staining of the mature zone in non-infected roots treated with 600 nM isoxaben or 0.01% DMSO.

Figure S4. DAB staining of *Heterodera schachtii*-infected roots treated with 600 nM isoxaben.

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