The atypical E2F transcription factor DEL1 modulates growth–defense tradeoffs of host plants during root-knot nematode infection

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In plants, growth–defense tradeoffs are essential for optimizing plant performance and adaptation under stress conditions, such as pathogen attack. Root-knot nematodes (RKNs) cause severe economic losses in many crops worldwide, although little is known about the mechanisms that control plant growth and defense responses during nematode attack. Upon investigation of Arabidopsis thaliana infected with RKN (Meloidogyne incognita), we observed that the atypical transcription factor DP-E2F-like 1 (DEL1) repressed salicylic acid (SA) accumulation in RKN-induced galls. The DEL1-deficient Arabidopsis mutant (del1-1) exhibited excessive SA accumulation in galls and is more resistant to RKN infection. In addition, excessive lignification was observed in galls of del1-1. On the other hand, the root growth of del1-1 is reduced after RKN infection. Taken together, these findings suggest that DEL1 plays an important role in the balance between plant growth and defense responses to RKN infection by controlling SA accumulation and lignification.

Salicylic acid (SA) is a key plant defense hormone required for immunity against pathogen infection1. Pathogens often induce SA accumulation both locally and systemically. This pathogen-induced SA is synthesized through the isochorismate pathway comprised of ISOCHORISMATE SYNTHASE 1 (ICS1), ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), and avrPphB SUSCEPTIBLE 3 (PBS3)2. A recent study has revealed that ICS1, EDS5 and PBS3 are necessary for pathogen-induced SA biosynthesis in plants2. SA accumulation leads to the activation of plant defense responses, including programmed cell death and systemic acquired resistance. High levels of SA accumulation result in changes in transcriptional activities of genes with antimicrobial activity such as the PATHOGENESIS-RELATED (PR) genes3. In Arabidopsis thaliana, SA-dependent transcriptional reprogramming requires NONEXPRESSER OF PR GENES 1 (NPR1), which was shown to be the bona fide SA receptor and also functions as a transcriptional co-activator4. Many studies have reported SA-induced defense responses upon infection by leaf-colonizing pathogens5, while little is known about the involvement of SA-induced defense responses in roots.

Several studies have shown that SA metabolism is also targeted by phytoparasitic root-knot nematodes (RKNs; Meloidogyne spp.) to ensure successful infection6. RKNs cause severe yield losses of many crop species in multiple regions of the world7. During RKN infections, juveniles invade plant roots and move toward the vascular cylinder. Upon reaching the vascular cylinder, RKNs induce the formation of galls, each containing several giant cells (GCs) which serve as a source of nutrients for the nematode until maturity. Since GCs result from nuclear divisions without cytokinesis (endoreduplication), it is thought that RKN is capable of manipulating the host cell-cycle machinery8. Biotrophic pathogens such as RKNs must keep the host cells alive while also suppressing host defense mechanisms. Repression of genes of the SA pathway was indeed observed in RKN-induced galls8–10. In addition, overexpression of NPR1 leads to the decrease in the number of galls and RKN fecundity during RKN infection11. On the other hand, overexpression of SA-degrading salicylate hydroxylase leads to reduced SA accumulation and greater susceptibility to RKN12.
SA accumulation contributes to plant defense responses, while excessive SA often leads to growth inhibition. Therefore, it is generally believed that host plants must balance growth and defense during pathogen infection. How this is accomplished is currently unknown. The Arabidopsis atypical transcription factor DP-E2F-1 (DEL1)/E2F2, a transcriptional repressor known to promote the onset of endoreduplication, has been shown to balance plant growth and defense via the SA response in leaves during pathogenic fungi infection. Here, we report that DEL1 represses excessive SA accumulation and root growth inhibition of host plants upon RKN infection. Furthermore, lignin deposition was also shown to be elevated in galls of the del1-1 knockout mutant. Our results suggest that DEL1 plays a role in balancing growth and defense in roots as previously reported in leaves.

Results

SA biosynthesis is enhanced in del1-1 galls. Chandran et al. showed that DEL1 acts as a transcriptional repressor of EDS5[10]. The enhanced resistance to fungi and the small stature phenotypes of del1-1 were shown to be SA-dependent. DEL1-mediated SA accumulation has been demonstrated in leaves[13], but it is unclear whether plants also balance growth–defense tradeoffs in the roots. To address this question, we subjected del1-1 to the RKN infection assay. We performed reverse transcription quantitative polymerase chain reaction (RT-qPCR) on genes related to SA biosynthesis in wild-type (WT) and del1-1 galls. EDS5 and PBS3 were up-regulated in the galls of del1-1 compared to the WT, while JCS1 did not show significant difference between transcript levels in del1-1 and WT galls (Fig. 1A). We next tested whether SA accumulation in del1-1 galls was higher than that in WT galls. Indeed, total SA accumulation in del1-1 galls was significantly higher than that in WT galls (Fig. 1B). On the other hand, total SA level in un-inoculated roots of del1-1 did not change significantly compared to that of WT (Fig. 1B). These results showed that DEL1 represses SA biosynthesis in RKN-induced galls.

Gall formation is compromised in del1-1. To determine whether the elevated SA levels activate the defense response, we analyzed the expression of the SA-dependent genes PR1, PR2, PR5 and NPR1 in galls. PR1 transcripts were not detected from the galls of neither WT nor del1-1 (Fig. S1), suggesting that PR1 is likely not involved in RKN infection. On the other hand, PR2 and PR5 were up-regulated in the galls of del1-1 compared to the WT, whereas NPR1 expression level did not change (Fig. S1). These results suggest transcript level of PR2 and PR5 may be modified by SA accumulation in the galls of del1-1. Previous studies have shown that SA signaling confers increased host plant resistance to nematode infection. To examine the functions of DEL1 in RKN-induced gall formation, we assayed gall formation frequencies and GC formation in del1-1. Gall numbers were reduced in the del1-1 mutant compared to that in the WT (Fig. 2A). Furthermore, histological analysis showed that GC areas were significantly reduced in del1-1 at 14 days post inoculation (dpi) by approximately one third of the WT value (Figs. 2B and S2), which is consistent with the results from a previous study. These results suggest that excessive SA accumulation from the loss-of-function of del1-1 mutation renders the host plant more resistant to RKN infection. Therefore it is possible that the endocycle machinery itself is involved in the RKN infection together with SA signaling, as de Almeida Engler et al. (2019) has shown that del1-1 mutant with defect of the endocyte machinery produced malformed giant cells.

DEL1 is involved in lignin deposition during RKN infection. In Arabidopsis roots, cell wall lignification is restricted to the xylem and the Casparian strip during normal development. Lignin contributes to the protection of plants from physical and chemical stresses, including pathogen challenges. Several studies have revealed that lignification can affect parasitic nematode infection rates. Galls during early stages were subjected to phloroglucinol-HCl staining to detect lignin, which was weakly stained in 3 dpi galls but became more prominent in 5 dpi galls in WT (Fig. 3A). In contrast, lignin was strongly stained in both 3 dpi and 5 dpi galls of del1-1 (Fig. 3A). In fact, 5 dpi galls of del1-1 showed stronger lignin staining than WT galls at 5 dpi (Fig. 3A). Non-galling parts of RKN-infected seedlings in both WT and del1-1 plants did not show lignin staining except for the vascular tissues (Fig. 3A). Next, we analyzed the transcript levels of lignin biosynthesis-related genes in galls by RT-qPCR. The expression levels of 4-coumarate: CoA ligase 1 (4CL1), 4CL2, cinnamate 4-hydroxylase (C4H), phenylalanine ammonia-lyase 1 (PAL1), PAL2, and cinnamate 4-hydroxylase (C4H) were significantly up-regulated in del1-1 galls compared to WT galls (Fig. 3B). These results indicate that DEL1 negatively regulates local lignin deposition during RKN infection.

del1-1 exhibits root growth inhibition after RKN infection. Chandran et al. (2014) showed that DEL1 controls rosette leaf size upon pathogen infection, while SA accumulation and lignin deposition are often associated with inhibition of growth in Arabidopsis. We therefore questioned whether DEL1 controls the balance between root growth and immunity after RKN infection. 10-day-old un-inoculated del1-1 seedlings did not show significant differences in root growth compared to WT (Fig. 4A,B), whereas the total root length of RKN-inoculated del1-1 seedlings at 7 dpi was significantly reduced compared to WT (Fig. 4A,C). These results indicate that DEL1 plays an important role in the balancing growth–defense tradeoffs in root during RKN-infection (Figs. 2 and 4).

Discussion

In plants, the activation of immunity is often inversely correlated with growth. Thus, the tradeoff between immunity and growth is thought to be important for survival of plants under biotic stresses. We demonstrated that knocking out DEL1/E2F2 leads to enhanced resistance and growth inhibition during RKN infection, likely due to excessive lignification and/or SA accumulation in RKN-induced galls (Fig. 5). Our RT-qPCR analysis showed that PR2 and PR5 were up-regulated in del1-1 galls, but PR1 was not (Fig. S1). This suggests a portion of the SA metabolism genes respond to RKN-induced SA accumulation in del1-1 galls. Since Fu et al. (2012) has shown that NPR1 protein level is up-regulated post-transcriptionally after SA treatment, NPR1 proteins are likely to be highly accumulated in del1-1 galls (Fig. S1). A number of studies have revealed that alterations in lignin biosynthesis...
result in changes in both growth and defense, while excessive lignification inhibits plant growth. In addition, transgenic and mutant plants with elevated basal SA levels also exhibit growth reduction. Moreover, recent studies have shown that SA accumulation in response to pathogen attack positively correlates with lignin deposition and acquisition of immunity. In light of these lines of evidence, DEL1 may mediate the balance between defense and growth by limiting SA accumulation during RKN infection in root. Chandran et al. have reported that del1-1 plants exhibit small rosette leaves and SA accumulation higher than that of WT regardless of fungal infection. On the other hand, our results showed that del1-1 exhibited root growth inhibition only in the presence of RKN infection (Fig. 4). SA accumulation in roots of del1-1 in the absence of RKN was not significantly different compared to that of WT (Fig. 1B), suggesting this difference in response between in leaves and in roots may come from differences of SA level in the absence of pathogen infection.

Although reduced parasitic nematode fecundity on del1-1 has been attributed to the deficiency in cell division during syncytia/gall formation, our results point to the additional possibility that the up-regulation of SA accumulation and lignification may also be contributing factors in gall formation efficiency. Further detailed analysis of the function of DEL1 in SA accumulation/lignification during RKN infection may provide insights into the mechanisms of balancing defense and growth in plants.
Figure 2. Gall formation is compromised in del1-1 mutants. (A) WT-normalized gall numbers in WT and del1-1 plants at 7 dpi (n = 15, WT; n = 16, del1-1). Sample sizes denote the number of petri dishes were tested. (B) Areas of GCs of 14 dpi galls in WT and del1-1 plants (n = 6). Sample sizes denote the number of galls. Means ± SD are shown. P values were analyzed by Student’s t-test.

Figure 3. Lignin accumulates specifically in del1-1 plants after RKN infection. (A) Phloroglucinol-HCl staining in non-infected regions of RKN-infected roots (control), 3 dpi and 5 dpi galls (5 individuals were observed with similar results). Top: WT. Bottom: del1-1. Scale bars = 100 µm. (B) RT-qPCR analysis of lignin synthesis-related genes in WT and del1-1 7 dpi galls. Values are normalized to expression levels in the WT. 4CL: 4-coumarate-CoA ligase, CAD: cinnamyl alcohol dehydrogenase, PAL: phenylalanine ammonia-lyase, C4H: cinnamate 4-hydroxylase. The experiment was repeated three times with similar results. Means ± SD are shown. ***P < 0.001 by Student’s t-test.
Methods

Plant materials and growth conditions. All Arabidopsis thaliana plants used in this study are of the Col-0 ecotype background. The mutant line, del1-1, was obtained from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis seeds were stratified for 2 days in 4 °C in the dark, then allowed to germinate and grown for 5 days on 0.25 × Murashige and Skoog (MS) salt mixture (Sigma), 0.5% (w/v) sucrose, and 0.6% (w/v) gellan gum at pH 6.4 under continuous light at 23 °C.

Figure 4. Root growth is inhibited in del1-1 mutants after RKN infection. (A) Representative images of WT and del1-1 seedlings with or without RKN inoculation. Top: 10-days-old non-inoculated seedlings. Bottom: RKN-inoculated seedlings at 7 dpi. (B) Total root length (primary root + lateral roots) of non-inoculated WT and del1-1 plants (n = 12, WT; n = 11, del1-1). (C) Total root length of RKN-inoculated WT and del1-1 plants (n = 18). Means ± SD are shown. Sample sizes denote the number of plants. The experiment was repeated three times with similar results. P values were analyzed by Student’s t-test.

Figure 5. Schematic model depicting the role of DEL1 during RKN infection. Proposed role for DEL1 as a mediator plant growth and defense in the RKN infection in Arabidopsis roots. In WT plants, DEL1 controls basal SA reeves and lignification in galls after RKN attack to balance plant growth and defense (green panel). Excessive SA accumulation and lignification due to misexpression of DEL1 contribute to plant defense after RKN attack, whereas the root growth is inhibited (orange panel).
Nematode preparation and inoculation. Root-knot nematodes (RKN, Meloidogyne incognita) were prepared aseptically as described previously. Briefly, 6- to 7-week-old tomato plants were inoculated at 3-day intervals for a total of four inoculations. Approximately 80,000 juveniles were used to inoculate each plant. The inoculated tomato plants were then transferred to a hydroponic system, and after 2 to 4 day intervals infective juveniles were collected from the hydroponic culture media. Six Arabidopsis seeds were sown on a MS plate. Five-days-old Arabidopsis seedlings were inoculated with approximately 80 nematodes per plant and incubated under short-day conditions (8 hours light/16 hours dark) at 25 °C. The roots of the seedlings were covered with black paper to mimic the dark underground environment in nature.

Evaluation of gall formation efficiency. Gall numbers were normalized for each petri dish of six seedlings to evaluate the gall formation efficiency (Data S1). The calculation procedure is as described below: (1) Galls number and germinated seedlings number were counted for each petri dish of six seedlings. (2) Gall number/seedling values were calculated. (3) Average values of galls/seedling in WT were calculated for each independent experiment to define the basal value (highlighted in blue in Data S1). (4) The galls/seedling values from WT and del1-1 were normalized to the basal value for each petri dish to calculate relative galls number. (5) Average values of relative gall numbers were calculated (highlighted in blue in Data S1). These values were used for Fig. 2A.

Phloroglucinol-HCl staining. Lignin was detected by the Wiesner test by immersing roots or galls of RKN-infected seedlings in phloroglucinol reagent [1% (w/v) phloroglucinol in 20% (v/v) HCl] for 5 minutes. Stained samples were then mounted in chloral hydrate solution (8g chloral hydrate, 2ml ultrapure water and 1ml glycerol). Samples were imaged with an Axio Imager M1 microscope (Carl Zeiss) mounted with a DP71 digital camera (Olympus).

Gene expression analysis. Total RNA was extracted from roots or galls (approximately 50mg fresh weight) using the RNeasy plant mini kit (Qiagen), then treated with Recombinant DNase I (Takara) according to the manufacturer’s instruction. First-strand cDNA was synthesized from 300ng of total RNA using PrimeScript RT Master Mix (Takara). The transcript level of target genes was assayed using FastStart Essential DNA Green Master (Roche) and the LightCycler 480 system (Roche). The thermal cycler program was 95 °C for 5 min followed by 55 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. GAPDH was used as the internal control and relative expression levels were calculated by the ΔΔCt method. Sequences of primers used are listed in Table S1. Each of the three biological replicates were performed in technical triplicates.

Histological analysis. Galls were dissected and transferred into 2% glutaraldehyde in 20 mM cacodylate buffer, pH 7.4, vacuum-infiltrated for 10 min twice, then incubated in 4 °C overnight. Samples were dehydrated in a graded ethanol series and embedded in Technovit 7100 (Kulzer) according to the manufacturer’s protocol. Sample blocks were sectioned to 3 μm thickness using an ultramicrotome (LEICA RM2255, Leica) and stained with 0.01% (w/v) toluidine blue O (W ALDECK) containing 1% (w/v) sodium borate decahydrate (Nacalai) for 5 minutes. Sample sections were then mounted in EUKITT (O. Kindler). Samples were imaged with an Axio Imager M1 microscope (Carl Zeiss) mounted with a DP71 digital camera (Olympus). Lengths and areas of the galls were quantified using ImageJ.

Quantification of total SA. Total SA (free SA plus SA glucosides) from Arabidopsis thaliana roots was extracted as previously described. Non-inoculated whole root systems (primary roots and lateral roots) of 12-days-old seedlings, or 7 dpi galls (more than 150 mg fresh weight) were collected. SA of the root exudates was determined by high performance liquid chromatograph–fluorescence detector (HPLC-FL) (LC-2000 plus and FP-2020 plus, Jasco) according to the previous report. Briefly, 20μL of sample was injected in water/methanol (20:80) eluent to be separated from other organic compounds by using a multi-mode column (Scherzo SM-C18, Imtakt, 2mm × 50mm). Fluorescence at 407 nm was monitored with 305 nm excitation for SA and o-anisic acid.

Statistical information. All P values were derived from two-sided Student’s t-tests or Tukey’s tests. All statistical tests and n numbers, including sample sizes or biological replications, are described in the figure legends.

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References
1. Vlot, A. C., Denspey, D. A. & Klessig, D. F. Salicylic Acid, a multifaceted hormone to combat disease. Annu. Rev. Phytopathol. 47, 177–206 (2009).
2. Rekhter, D. et al. Isochorismate-derived biosynthesis of the plant stress hormone salicylic acid. Science 365, 498–502 (2019).
3. Wang, D., Amornsiripanitch, N. & Dong, X. A Genomic Approach to Identify Regulatory Nodes in the Transcriptional Network of Systemic Acquired Resistance in Plants. PLoS Pathog. 2, e123 (2006).
4. Wu, Y. et al. The Arabidopsis NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid. Cell Rep. 1, 639–647 (2012).
5. Palmer, I. A., Shang, Z. & Fu, Z. Q. Salicylic acid-mediated plant defense: Recent developments, missing links, and future outlook, Front. Biol. 12, 258–270 (2017).
6. Favary, B., Quentin, M., Jaubert-Possamai, S. & Abad, P. Gall-forming root-knot nematodes hijack key plant cellular functions to induce multinucleate and hypertrophied feeding cells. J. Insect Physiol. 84, 60–69 (2016).
7. Jones, J. T. et al. Top 10 plant-parasitic nematodes in molecular plant pathology: Top 10 plant-parasitic nematodes. Mol. Plant. Pathol. 14, 946–961 (2013).
8. Barcala, M. et al. Early transcriptomic events in microdissected Arabidopsis nematode-induced giant cells. Plant. J. 61, 698–712 (2010).
32. Yuan, W., Allasia, V., Fu, Z. Q., Xie, M., Ma, R., Tang, Y., Jirage, D., Bowling, S. A., Wuyts, N., Lognay, G., Swennen, R., & De Waele, D. Nematode infection and reproduction in transgenic and mutant Arabidopsis. BMC Res. Notes 4, 231 (2011).

28. Fu, Z. Q., Xie, M., & Gheysen, G. The Jasmonate Pathway Is a Key Player in Systemically Induced Defense against Root Knot Nematodes in Rice. Plant. Physiol. 157, 305–316 (2011).

Chandran, D. et al. Atypical E2F Transcriptional Repressor DEL1 Acts at the Intersection of Plant Growth and Immunity by Controlling the Hormone Salicylic Acid. Cell Host Microbe 15, 506–513 (2014).

Molinari, S., Fanelli, E., & Leonetti, P. Expression of tomato salicylic acid (SA)-responsive pathogenesis-related genes in transgenic and mutant Arabidopsis. Mol. Plant Pathol. 15, 255–264 (2014).

Wubben, M. J. E., Jin, J., & Baum, T. J. Cyst Nematode Parasitism of Arabidopsis thaliana Is Inhibited by Salicylic Acid (SA) and Elicits Uncoupled SA-Independent Pathogenesis-Related Gene Expression in Roots. MPMI 21, 424–432 (2008).

de Almeida Engler, J., et al. CCR52 and DEL1 genes are key components of the endocycle in nematode-induced feeding sites. Endocycle in nematode feeding sites. Plant. J. 72, 185–198 (2012).

Barros, J., Serk, H., Granlund, I., & Pesquet, E. The cell biology of lignification in higher plants. Ann. Bot. 115, 1033–1047 (2015).

Fujimoto, T., Mizukubo, T., Abe, H., & Seo, S. Sclareol Induces Plant Resistance to Root-Knot Nematode Partially Through Ethylene-Dependent Enhancement of Lignin Accumulation. MPMI 28, 398–407 (2015).

Huang, W. K., Jirage, D., Gheysen, G., & Kyndt, T. Thiamine-induced priming against root-knot nematode infection in rice involves lignification and hydrogen peroxide generation: VB1-induced priming against root-knot nematode. Mol. Plant Pathol. 17, 614–624 (2016).

Veronico, P. et al. Changes in lignin biosynthesis and monomer composition in response to benzothiadiazole and root-knot nematode Meloidogyne incognita infection in tomato. J. Plant. Physiol. 230, 40–50 (2018).

Wuets, N., Lognay, G., Swennen, R., & De Waele, D. Nematode infection and reproduction in transgenic and mutant Arabidopsis and tobacco with an altered phenylpropanoid metabolism. J. Exp. Botany 57, 2825–2835 (2006).

Bowling, S. A. et al. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell 6, 1845–1857 (1994).

Ijirage, D. et al. Constitutive salicylic acid-dependent signaling in cpr1 and cpr6 mutants requires PAD4. Plant J. 26, 395–407 (2002).

Mauch, F. et al. Manipulation of salicylate content in Arabidopsis thaliana by the expression of an engineered bacterial salicylate synthase: Manipulation of salicylate content in Arabidopsis. Plant. J. 25, 67–77 (2006).

Zhong, R., Demura, T., Ye, Z., & H. SND1, a NAC Domain Transcription Factor, Is a Key Regulator of Secondary Wall Synthesis in Fibers of Arabidopsis. Plant Cell 18, 3158–3170 (2006).

Zhong, R., Richardson, E. A., & Ye, Z. H. The MYB46 Transcription Factor Is a Direct Target of SND1 and Regulates Secondary Wall Biosynthesis in Arabidopsis. Plant Cell. 21, 2776–2792 (2009).

Zhou, J., Lee, C., Zhong, R., & Ye, Z. H. MYB58 and MYB63 Are Transcriptional Activators of the Lignin Biosynthetic Pathway during Secondary Cell Wall Formation in Arabidopsis. Plant Cell. 21, 248–266 (2009).

Fu, Z. Q. et al. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 466, 228–232 (2012).

Xie, M. et al. Regulation of Lignin Biosynthesis and Its Role in Growth-Defense Tradeoffs. Front. Plant. Sci. 9, 1427 (2018).

Ma, R. et al. AP2/ERF Transcription Factor, Ito049, Positively Regulates Lignan Biosynthesis in Isatis indigotica through Activating Salicylic Acid Signaling and Lignin/Lignin Pathway Genes. Front. Plant. Sci. 8, 1361 (2017).

Tang, Y. et al. Cotton WATs Modulate SA Biosynthesis and Local Lignin Deposition Participating in Plant Resistance Against Verticillium dahliae. Front. Plant. Sci. 10, 526 (2019).

Yuan, W. et al. Maize phenylalanine ammonia-lyases contribute to resistance to Sugarcane mosaic virus infection, most likely through positive regulation of salicylic acid accumulation. Molecular Plant Pathology 12817 (2019).

Zhang, Z. et al. Cotton plant defence against a fungal pathogen is expanded by enhancing BLADE-ON-PETIOLE1 expression beyond lateral-organ boundaries. Commun. Biol. 2, 238 (2019).

Nishiyama, H. et al. Protocol for root-knot nematode culture by a hydroponic system and nematode inoculation to Arabidopsis. Japanese J. Nematology 45, 45–49 (2015).

Allasia, V. et al. Quantification of Salicylic Acid (SA) and SA-glucosides in Arabidopsis thaliana. BIO-PROTOCOL 8 (2018).
