A broadly conserved NERD genetically interacts with the exocyst to affect root growth and cell expansion

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Abstract
The exocyst, a conserved, octameric protein complex, helps mediate secretion at the plasma membrane, facilitating specific developmental processes that include control of root meristem size, cell elongation, and tip growth. A genetic screen for second-site enhancers in Arabidopsis identified NEW ENHANCER of ROOT DWARFISM1 (NERD1) as an exocyst interactor. Mutations in NERD1 combined with weak exocyst mutations in SEC8 and EXO70A1 result in a synergistic reduction in root growth. Alone, nerd1 alleles modestly reduce primary root growth, both by shortening the root meristem and by reducing cell elongation, but also result in a slight increase in root hair length, bulging, and rupture. NERD1 was identified molecularly as At3g51050, which encodes a transmembrane protein of unknown function that is broadly conserved throughout the Archaeplastida. A functional NERD1–GFP fusion localizes to the Golgi, in a pattern distinct from the plasma membrane-localized exocyst, arguing against a direct NERD1–exocyst interaction. Structural modeling suggests the majority of the protein is positioned in the lumen, in a β-propeller-like structure that has some similarity to proteins that bind polysaccharides. We suggest that NERD1 interacts with the exocyst indirectly, possibly affecting polysaccharides destined for the cell wall, and influencing cell wall characteristics in a developmentally distinct manner.

Keywords: Arabidopsis, cell elongation, cell wall, exocyst, genetic interaction, root development, root hair, root meristem, secretory pathway, tip growth.

Introduction
The secretory system in plants is a fundamental determinant of plasma membrane composition and cell wall formation (Luschnig and Vert, 2014; Ebine and Ueda, 2015; Kim and Brandizzi, 2016). Consequently, secretory events drive cell growth and morphogenesis, and influence plant development. For example, selective localization of secretion to specific regions of the cell periphery is essential for polarized growth of pollen tubes, enabling sperm cell delivery and sexual reproduction in flowering plants. Additionally, secretion of substances into the apoplast and delivery of receptors and transporters to the plasma membrane allow for intracellular communication and coordination. Ultimately, the secretory system’s intimate influence on the plasma membrane and extracellular activities facilitates responsiveness and survival within variable abiotic and biotic environments. However, it remains unclear how the secretory process in plants is spatially...
and temporally regulated to direct particular cargos to specific locations of the plasma membrane, cell wall, or apoplast at the appropriate time. Both the conventional secretory system, i.e. vesicular transport from endoplasmic reticulum to Golgi to plasma membrane, and non-conventional pathways are involved (Drakakaki and Dandekar, 2013; Robinson et al., 2016; van de Meene et al., 2017), but how these pathways are tailored to the dynamic requirements of different cell types is only beginning to be revealed.

The exocyst, an evolutionarily conserved octameric complex, tethers secretory vesicles to specific sites on the plasma membrane prior to exocytosis and modulates secretory activity to achieve an array of specialized functions. In plants, components of the exocyst have been implicated in a range of processes including pollen tube germination and growth (Cole et al., 2005; Hála et al., 2008; Li et al., 2010), cytokinesis (Fendrych et al., 2010; Rybak et al., 2014), secondary cell wall deposition during tracheary element development (Li et al., 2013; Oda et al., 2015), hypocotyl elongation in etiolated seedlings (Hála et al., 2008), determination of meristem size and cell elongation during primary root growth (Cole et al., 2014), Casparian strip formation (Kalmbach et al., 2017), localized disposition of seed coat pectin (Kulich et al., 2010), callose deposition in trichomes (Kulich et al., 2015), and the polar growth of root hairs (Wen et al., 2005; Syn et al., 2006).

The regulation, assembly, and functioning of the exocyst complex in non-plant eukaryotes has been linked to its interactions with small GTPases of the Rho, Rab, and Rab families (Mukherjee et al., 2014), membrane phospholipids (Thapa et al., 2012; Pleskot et al., 2015), plasma membrane scaffolding proteins (Liu and Novick, 2014), and the actin cytoskeleton (Jin et al., 2011; Liu et al., 2012). This interactive milieu helps define exocyst function in yeast and mammals, providing post-translational regulation of key secretory events (Wu and Guo, 2015; Pleskot et al., 2015). In plants, the molecular mechanisms that integrate the exocyst into distinct secretory processes are less well understood. One regulatory mechanism unique to plants is the proliferation and diversification of homologs of the exocyst subunit EXO70 (23 in Arabidopsis), which is hypothesized to allow for specification of particular exocyst functions (Syn et al., 2006; Li et al., 2010; Cvrčková et al., 2012; Vukašinović and Žárský, 2016). In support of this hypothesis, different EXO70 paralogs have been associated with specific cellular processes: EXO70B1 with autopagy (Kulich et al., 2013), EXO70F1 with arbuscular mycorrhizal symbiosis (Zhang et al., 2015), and EXO70E with the EXPO secretory pathway (Poulsen et al., 2014). Furthermore, in growing pollen tubes, members of the EXO70A, EXO70B and EXO70C subgroups show differential localization patterns and apparent activities (Sekereš et al., 2017; Syn et al., 2017). Other factors that help regulate the exocyst in specific developmental contexts are the scaffolding protein Interactor of Constitutive active ROPs 1 (ICR1) in roots (Lavy et al., 2007); the phosphoinositide PIP2 in pollen tubes (Bloch et al., 2016); ROP2 GTPase (with its effector RIC7) in stomata (Hong et al., 2016); and the combined activities of VETH1–VETH2–COG2 and cortical microtubules in xylem cells (Oda et al., 2015). However, given the breadth of functions known for the plant exocyst, other factors are likely to be involved, including cellular components that interact with the exocyst indirectly, e.g. by enhancing the activity of an exocyst-trafficked protein.

To advance the investigation of exocyst-mediated secretory events in plants, we performed a mutagenesis screen to identify interactors linked to the exocyst’s role in Arabidopsis root growth. In this screen, we identified NEW ENHANCER OF ROOT DWARFISM (NERD1), a protein of unknown function that, based on genetic interaction data, acts with the exocyst to facilitate root and hypocotyl elongation and to influence root hair morphology. NERD1 is expressed throughout the plant, suggesting a potential role beyond the root and hypocotyl. NERD1 homologs are found throughout the plant kingdom and beyond. Interestingly, the functional interaction of the exocyst with NERD1 is likely to be indirect, and varies dependent on developmental context. We speculate that NERD1 is involved in the modification of cell wall polysaccharides that are important for cell wall expansion and are a cargo for exocyst-mediated transport to the apoplast.

### Materials and methods

#### Plant materials and growth conditions

Lines of Landsberg erecta-0 and Columbia-0 ecotype of Arabidopsis with T-DNA insertions were obtained from the SALK Institute (Alonso et al., 2003): nerd-1-2 (At3g51050, SALK 018060C); nerd-1-3 (At3g51050, SALK 051660); exo70A1-2 (At5g3540, SALK 135462); sec8-3 (At3g10380, SALK 026204); sec8-4 (At3g10380, SALK 118129); sec8-6 (At3g10380, SALK 091118); and myo XI-K (At5g20490, SALK 067972). The exo84h-1 line was a GABI-Kat line (Rosso et al., 2003; Fendrych et al., 2010). The EXO84h-GFP and GFP-SEC8 lines were previously described (Fendrych et al., 2010). The nerd-1-1 mutant was generated in an ethyl methanesulfonate (EMS) screen that treated ~5000 sec8-6 seeds with 0.2% EMS for 15 h. M2 generation seed from 4500 M1 plants was collected in pools derived from 16 plant lots. The effectiveness of the mutagenesis was verified by observing greater than 64% of M2 plants with one-quarter aberrant seed, with the gene mutation rate estimated at 1/3000.

Arabidopsis seeds were surface-sterilized, stratified at 4 °C for 3–5 d, and planted on growth medium (1× MS, 2% (w/v) sucrose, and vitamins in 1% (w/v) Bacto-agar) or soil as previously described (Cole et al., 2005). Plants were grown in a climate chamber at 22 °C under long-day conditions (16 h of light per day; 7500 lx), with the exception of those used in hypocotyl elongation experiments. For these, seeds were placed in a lighted incubator at 228°C for 2–4 h to stimulate germination and then wrapped in foil, oriented vertically, and placed in a dark box in a 22 °C incubator. After 5 d in the dark, digital images were captured and hypocotyl lengths measured.

To evaluate the effect of Endosindm2, three groups were germinated on MS plates: seedlings that were homozygous for nerd-1-2; nerd-1-2 siblings complemented by NERD1–green fluorescent protein (GFP); and Col-0 plants. Plants of each genotype were transferred approximately 3 d after germination to plates containing 0, 20, or 40 μM Endosindm2 (ES2), and grown for an additional 4 d before imaging to determine primary root growth rates. DMSO-dissolved ES2 (or DMSO alone as a control, at 0.5% (v/v)) was added to media during plate preparation.

#### High-throughput sequencing and analysis

A plant homozygous for nerd-1-1 and lacking a sec8 allele in a Col-0 background was backcrossed to Ler-0, and the progeny were self-crossed to generate an F2 population. Pooled genomic DNA from 150 F2 plants with the nerd phenotype (i.e. homozygotes) was sequenced via an Illumina HiSeq 2000 to generate 58 million paired-end reads. SHOR-Enmap software (Schneeberger et al., 2009) was used to align the
reads to the Arabidopsis genome and assess the frequency of Col (the mutagenized parent) and Ler single nucleotide polymorphisms (SNPs) across the population. All variant SNPs in the ~200 kb region of chromosome 3 harboring nerd1-1 (Supplementary Fig. S1 at JXB online) were then searched against genes to identify candidate mutations with likely deleterious effects.

Genetic and molecular analyses

DNA extraction from leaves and PCR genotyping for mutants containing T-DNA insertions was performed as previously described (Cole et al., 2005). Primers used in PCR and RT-PCR are shown in Supplementary Table S1. PCR-based genotyping to detect the EMS-generated nerd1-1 mutation required use of the restriction enzyme AvaII after amplification, as a target cleavage site in the At3g51050 genomic sequence was eliminated by the G→A transition in the mutant. To evaluate expression via RT-PCR, roots from approximately 50 10-day-old seedlings of each genotype were harvested from plates and frozen in liquid nitrogen. RNA was extracted from the pooled sample for each genotype using a phenol-chloroform procedure, followed by DNase treatment. First strand cDNA synthesis was performed using Superscript II as per the manufacturer’s specifications (Thermo Fisher Scientific), followed by removal of RNA with RNaseH. The cDNA was used as a template for PCR with primer pairs that amplified the sequence to the 5′ of the mutations, to the 3′ of the mutations, or spanning the sites of the mutations. Primers for ACTIN2 were included as an internal control.

Generation of NERD1–GFP and imaging

A 5597-bp-long genomic fragment encompassing the NERD1 gene along with its putative promoter was PCR amplified from the genomic DNA using KOD Hot Start high-fidelity DNA polymerase (Novagen) and cloned into a modified pMDC32 plasmid using Styl and PstI sites. Enhanced GFP (EFGP; Clontech) cDNA was added downstream from the NERD1 open reading frame (ORF) to yield pMDC-NERD1-GFP. The plasmid was mobilized into Agrobacterium tumefaciens strain GV3101. Transient expression or coexpression of NERD1 construct and fluorescent Golgi markers in Nicotiana benthamiana leaf epidermal cells was performed by co-infiltrating with Agrobacterium strains carrying NERD1-GFP and either STlmd-YFP or NAG-mTurq (Peremyslov et al., 2012) at concentrations equal to 0.2 OD600. Imaging was conducted 2 d post-infiltration. Leaf fragments were immersed in water and observed using a Zeiss LSM 780 NLO confocal microscope equipped with a Plan-Apochomat ×63 1.4 NA lens. mTurquoise, GFP, and mCherry were excited with the 405 nm diode laser line, 488-nm argon laser line, or 561-nm He–Ne laser line, respectively. For the simultaneous visualization of two fluorophores, dual channel acquisition of signal for either GFP and mTurquoise or GFP and mCherry was performed sequentially to minimize crosstalk. For Brefeldin A (BFA) sensitivity, Arabidopsis seedlings expressing fluorophore-tagged proteins were treated with 50 μM BFA for 90 min, and the BFA-sensitive endosome compartments were imaged in root epidermal cells.

Evaluation of cortical cell files and root growth parameters in nerd1 mutants using confocal microscopy was performed as previously described (Cole et al., 2014). Briefly, images of roots grown on vertical plates were captured on day 5 and day 7 after germination to determine root growth rates. The 7-day-old seedlings were stained with propidium iodine and then imaged with a Zeiss LSM 780 NLO confocal microscope equipped with a Plan-Neofluor 100×1.35 NA oil objective. Measurements (e.g. root lengths, hypocotyl lengths, root hair dimensions, and root cortical cell lengths and widths) from confocal digital images were achieved using ImagePro analysis software (MediaCybernetics). Transmitted light images of hypocotyl and root hair specimens were captured with a Leica DFC 295 digital camera attached to a Zeiss Stemi SV11 dissecting microscope, utilizing Leica Application Suite v3.8.

Results

Screening for exocyst interactors

Mutations in genes encoding components of the exocyst complex in Arabidopsis result in root growth defects that vary from a mild decrease in growth rate in some mutants (e.g. sec8-6 and exo70A1) to severe dwarftism in others (e.g. exo84b-1 and sec8-3) (Cole et al., 2014). Additionally, mutations of some exocyst components reduce the length of root hairs (Snyek et al., 2006). We reasoned that a protein that interacts with the exocyst could be revealed if its mutation accentuated the root growth defect of an exocyst mutation that by itself results in only a mild phenotype. Therefore, we screened an EMS-treated population of seedlings homozygous for the mild sec8-6 mutation in a Col-0 background to identify such second-site enhancers.

Plants from M2 pools exhibiting both short roots and aberrant root hairs—dubbed the new enhancer of root dwarfism (nerd) phenotype—were outcrossed to a wild-type line, self-pollinated, and screened again for the phenotype in ~1/16 of the progeny, as expected for second site enhancers. After screening 45 pools, we recovered exactly one such mutation, nerd1-1, which, when combined with sec8-6, leads to profound dwarfism throughout the plant and shorter primary roots (~25% of wild-type length, with some demonstrating terminated growth). In addition, root hairs in nerd1 sec8-6 double mutants are occasionally misshapen (Supplementary Fig. S2). To verify that the genetic interaction was not specific to a particular SEC8 allele, the nerd1-1 mutation (isolated after a series of backcrosses to Col-0) was combined with another mild allele, sec8-4, yielding a similar result (Fig. 1A). Intriguingly, initial observations indicated that nerd1 plants, in the absence of a sec8-6 or sec8-4 mutation, have a similar, but less severe phenotype—e.g. root length ~75% of wild-type, and less frequently misshapen root hairs.

Molecular identification of NERD1

To identify the nerd1-1 lesion, we used high-throughput sequencing of a pooled population of mutant plants to identify a ~200 kb region of chromosome 3 tightly linked to nerd1 (Supplementary Fig. S1). This region encompassed ~65 protein-coding genes, only two of which harbored putative EMS-generated G→A mutational differences from the Col-0 reference sequence linked to nerd1-1. Both mutations were validated via Sanger sequencing. The best candidate for nerd1-1 appeared to be a change in a conserved splice acceptor site at the ninth exon of At3g51050 (Fig. 1B). To confirm the molecular identity of NERD1, two independent T-DNA insertion alleles in At3g51050 (Fig. 1B) were obtained from the Salk mutant collection, both of which were associated with short root and root hair defects. Subsequent complementation tests between heterozygotes for all three alleles showed the nerd1 root growth and root hair phenotype appearing in approximately 25% of the progeny, verifying that the two insertion alleles (designated nerd1-2 and -3) were indeed inactivating this same locus affected in the original nerd1-1 line, and proving that At3g51050 corresponds to the NERD1 gene. This
segregation pattern further shows that \textit{nerd1} mutants do not have a significant gametophyte-derived transmission defect. Notably, pollen is the developmental stage, across 105 stages assessed in the Genevestigator database (Grennan, 2006), associated with lowest expression of At3g51050. The absence of a significant transmission defect in \textit{nerd1} mutants, and the low level of expression, suggests that \textit{NERD1} function is not as central to polarized growth in pollen tubes as it is in root hairs.

In addition to the similar phenotypic severity of each of the three alleles, RT-PCR assays suggested that all three were nulls, each generating aberrant transcripts that likely produce non-functional protein. \textit{NERD1} transcripts in \textit{nerd1-1}
homologs were shorter than wild-type in the region spanning the point mutation, and thus were likely mis-spliced, whereas transcripts in nerd1-2 and -3 were detected upstream, but not downstream from their respective T-DNA insertions (Supplementary Fig. S3).

The 698 amino acid-long NERD1 sequence is broadly conserved in plants, and homologs are detectable in non-plant species (Supplementary Dataset S1). The Gramene EnsemblePlants database (Kersey et al., 2016) identifies NERD1 homologs in 42 Viridiplantae species, primarily angiosperms, but also including more distantly related Archaeplastida species, including members of Bryophyta (Physcomitrella patens), Lycopodiophyta (Selaginella moellendorffii), and Chlorophyta (Chlamydomonas reinhardtii and Ostreococcus lucimarinus). In Arabidopsis and other Viridiplantae species, NERD1 is identified as a single copy gene. Certain regions of NERD1 also show notable similarity to proteins in both Metazoan and Amoebozoan species (Supplementary Dataset S1).

Protein modeling software was used to predict potential structural characteristics of NERD1 (Fig. 1C; Supplementary Table S2; Supplementary Figs S4 and S5; Supplementary Datasets S2 and S3). Arabidopsis NERD1 contains an N-terminal signaling peptide that is well conserved across all Datasets S2 and S3). Arabidopsis NERD1 contains an N-terminal signaling peptide that is well conserved across all datasets. The predicted β-propeller structure of NERD1 folds into a globular protein with a β-propeller structure: seven predicted β-sheets arranged radially and pseudo-symmetrically around a central axis (Fig. 1D–E; Supplementary Fig. S5). β-Propellers are widely used as structural scaffold, providing a surface for ligand binding and enzymatic activity (Kopec and Lupas, 2013). In addition, NERD1’s β-propeller contains a putative calcium-binding pocket. The predicted tertiary structure resembles templates for some pyrroloquinoline quinone-dependent enzymes (e.g. alcohol dehydrogenases) and, intriguingly, shares similarity to proteins that interact with polysaccharides or glycoproteins, including lectins, integrins, carbohydrate binding proteins, and perhaps most notably, some pectin lyases and xyloglucanases (Supplementary Dataset S2). Overall, however, the full length of NERD1 is not strictly homologous to members of any known protein family. Although the exact 3D structure and molecular function of NERD1 remain uncertain, these predictions raise the intriguing possibility that NERD1 is an integral membrane protein that interacts with polysaccharides, potentially in a calcium-dependent manner. Notably, NERD1 (At3g51050) mRNA is expressed throughout most of the Arabidopsis plant, with little change induced by developmental or environmental variables (Grennan, 2006), suggesting that NERD1 is a component of most plant cells.

Localization of NERD1

Previous large-scale proteomic analyses detect NERD1 at the plasma membrane and/or Golgi (Mitra et al., 2009; Zhang and Peck, 2011; Parsons et al., 2012; Heard et al., 2015). To validate and refine these findings, a genomic clone harboring the native promoter and complete ORF of NERD1 was tagged with GFP at the 3′ end and expressed transiently in N. benthamiana or used to generate transgenic Arabidopsis plants. Confocal microscopy of leaf epidermis cells of N. benthamiana revealed that the tagged protein is present in small motile bodies of ca. 1 μm in diameter that resembled Golgi stacks (Fig. 2A). To investigate the nature of these bodies, we examined cells of N. benthamiana co-expressing NERD1–GFP and one of two different Golgi markers: STtmd–Cherry or an N-acetylgalactosaminyl transferase fused to fluorescent protein mTurquoise (NAG–mTurq) (Peremyslov et al., 2012). In all cells examined, the GFP signal co-localized with these Golgi markers, indicating that NERD1 is primarily present in Golgi (Fig. 2C–E; Supplementary Fig. S6).

The functionality of the NERD1–GFP fusion protein was validated by genetic complementation. To this end, the NERD1–GFP expression cassette was stably transformed into an Arabidopsis nerd1-2 heterozygote line, which was subsequently self-crossed. A progeny plant homozygous for the nerd1-2 mutation but phenotypically wild-type was identified and self-crossed. PCR genotyping verified that all the resultant seedlings were homozygous for the nerd1-2 mutation. One-fourth of these plants (13 of 52) exhibited the nerd root phenotype (shorter roots: 7.6 ± 1.3 mm versus wild-type 13.0 ± 2.6 mm, t-test P<10−12; and altered root-hair morphology); all plants exhibiting the nerd phenotype were negative for the cassette presence and NERD1–GFP expression. In contrast, the fusion cassette was present and expressed in all the seedlings that were phenotypically wild-type, indicating that it provides a functional NERD1 protein. Confocal microscopy revealed that the NERD1–GFP in a nerd1-2 mutant background was localized to mobile punctate structures in the cytoplasm (Fig. 2B), similar to the observations in N. benthamiana, and consistent with NERD1 localization in the Golgi. To further validate association of NERD1–GFP with Golgi, we investigated sensitivity of the fluorescent bodies to BFA, which disrupts Golgi architecture and induces formation of an endoplasmic reticulum (ER)–Golgi hybrid compartment (Ritzenthaler et al, 2002). Arabidopsis seedlings stably expressing either NERD1–GFP or, as a control, NAG–mTurq, were incubated with this drug. As expected, BFA treatment resulted in formation of a typical BFA compartment marked by either NAG–mTurq or NERD1–GFP in each line (Supplementary Fig. S7), strongly supporting the Golgi residence of NERD1–GFP.

The localization of NERD1–GFP in the Golgi is notably distinct from that observed for components of the exocyst at the plasma membrane and in the cytoplasm (Fendrych et al., 2010, 2013; Li et al., 2013; Oda et al., 2015). This suggests that the interaction between NERD1 and the exocyst is not a direct interaction at the plasma membrane, as we had initially hypothesized. One possibility is that NERD1 in the Golgi is
important for correct transit of exocyst components to the plasma membrane. Thus, we tested whether the nerd1 mutation causes a mislocalization of the exocyst by imaging GFP-labeled exocyst components. Both EXO84–GFP and SEC8–GFP localization patterns at the plasma membrane in nerd1 mutant roots were indistinguishable from their localization in wild-type controls (Fig. 3A and B, and 3C and D, respectively). Thus, the nerd1 mutant root phenotype is not explained by a mislocalization of the exocyst. The converse was also considered, i.e. do exocyst mutations result in altered localization of NERD1? Observation of NERD1–GFP in the roots of exo70A1 and exo84b mutants revealed that NERD1 localization (i.e. in punctate structures within the cytoplasm) was not altered by mutation of these exocyst components (Fig. 3E–H). These data further argue that the genetic interaction of NERD1 and exocyst mutants is indirect.

Genetic interactions with NERD1 mutants depends on developmental context

The apparent absence of co-localization of NERD1 and the exocyst motivated a quantitative assessment of their phenotypes and genetic interactions in three distinct developmental contexts. Mutations of NERD1 combined with mutations of exocyst components were examined for their effects on primary root growth, cell elongation in etiolated hypocotyls, and the polarized growth of root hairs. Intriguingly, while all three developmental contexts involve some degree of cell expansion and are impacted by both NERD1 and the exocyst, as detailed below, the specific genetic interactions varied, depending upon context.

Growth of primary root and etiolated hypocotyl

Primary root growth was examined in plants harboring the nerd1-1 or nerd1-3 mutation in combination with a mutation in an exocyst component, exo70A1 or sec8-4. A comparison of sibling plants confirmed that the primary root growth defect was more severe in the double mutants than in either of the single mutants (Fig. 4A). Additive and multiplicative models were used to predict the severity of the root growth defect that would be observed if the mutations were non-interacting (Hála et al., 2008; Mani et al., 2008). The observed growth rate defect in the double mutants was much more severe than predicted by either model, verifying a synergistic interaction between NERD1 and the exocyst in root growth. To further substantiate the functional interaction between NERD1 and the exocyst, nerd1-2 mutants were treated with the chemical Endosidin2 (ES2), which inhibits exocyst

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Fig. 2. NERD1–GFP localizes to the Golgi. (A) NERD1–GFP in Nicotiana benthamiana leaf cells. (B) Root tip of a NERD1-GFP-complemented nerd1-2 mutant. (C–E) Co-localization of NERD1 with the Golgi marker: (C) STtmd::mCherry (sialyltransferase transmembrane domain), (D) NERD1–GFP, and (E) merged image. Scale bars: 5 μm (A, C, D, E) and 20 μm (B).
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As expected, root growth rates of control nerd1-2 plants harboring the NERD1–GFP construct were not significantly different from those of Col-0 seedlings grown on media containing 0, 20, or 40 μM ES2 (Fig. 4B). In contrast, nerd1-2 homozygotes are significantly more sensitive to the effect of ES2 on root growth rate, at both 20 and 40 μM ES2. Thus, similar to the genetic interaction, the effect of pharmacological inhibition of the exocyst on root growth in nerd1-2 mutants is more than would be predicted by multiplicative or additive models (Fig. 4B).

The primary root growth defect in exocyst mutants is due to both a reduced number of cells dividing in a shorter meristem, and a slower rate of cell expansion in the elongation zone (outside the meristem) (Cole et al., 2014). Given the functional interaction between NERD1 and the exocyst in the primary root, we were curious to know if nerd1 mutant effects could be attributed to one or both of these underlying mechanisms. Consequently, cortical cell files in the root tips of nerd1 mutants were examined by confocal microscopy and compared with those in the root tips of Col-0 and exo84b (an exocyst mutant with a severe root growth defect) grown on the same vertical plates (Table 1). Similar to exocyst mutants with severe root growth defects (e.g. exo84b-1) (Cole et al., 2014), the reduced primary root growth in nerd1 mutants arises from both less cell elongation, leading to shorter mature cells, and a reduced number of cells dividing in shorter meristems. These defects were quantitatively similar for all three nerd1 alleles, and less severe than in exo84b-1. Notably, mature cortical cell widths in nerd1 mutants are similar to those in Col-0, indicating that the nerd1 defect is specific to cell elongation. This contrasts with exocyst mutant cells, in which overall mature cortical cell size, both length and width, is reduced. Thus, NERD1 appears to be more specifically involved in longitudinal expansion of lateral walls in the root elongation zone.

Elongation of the hypocotyl in etiolated seedlings, in contrast to the more complex process of root growth, is due solely to cell elongation, thereby providing a second and more specific system to evaluate the genetic interaction of NERD1 and exocyst mutants in cell elongation. Hypocotyl lengths and epidermal cell lengths in the hypocotyls of 5-day-old dark grown nerd1-3 sec8-4 double mutant seedlings were evaluated and compared with similar measurements in single mutant and wild-type siblings (Supplementary Fig. S8). As in roots, nerd1-3 and sec8-4 interact synergistically to reduce hypocotyl lengthening (Supplementary Fig. S8B). Furthermore, the effect on the hypocotyl was associated with a synergistic defect in cell elongation (Supplementary Fig. S8C), again similar to the results in the primary root. These data are consistent with expression data pointing to a role for NERD1 and the exocyst in cell growth throughout the plant.

Fig. 3. Subcellular localizations of NERD1 and exocyst markers are independent of each other. The localization of exocyst markers to the outer surface of root epidermal cells of nerd1-2 mutants (A, C) is similar to that in wild-type siblings (B, D). Conversely, the predominant localization of NERD1–GFP in the cytoplasm of root epidermal cells of exocyst mutants (E, G) is similar to that of wild-type siblings (F, H). Shown are epidermal cells in the root transition zone (A–D, G, H) and meristem (E, F). Confocal images provide radial longitudinal sections through the center of the root (A, B, E, F) in which the upper portion of cells shown are on the root surface. In tangential sections (C, D, G, H) parallel with the root surface the lateral walls of the epidermal cells are shown. Scale bars: 20 μm. (This figure is available in colour at JXB online.)
One phenotype leading to selection of the initial nerd1 allele was altered root hair morphology. Short root hairs are characteristic of several exocyst mutants (e.g. exo70A1 and exo84b) (Synek et al., 2006), whereas root hairs that are wild-type in length are observed in other exocyst mutants (e.g. sec8-4). As in the primary root, abrogating exocyst function does not alter NERD1 localization patterns in the root hair (Fig. 5A, B).
Nevertheless, comparison of root hairs in exo70A1 nerd1-3 double mutants and sibling single mutants revealed a genetic interaction (Fig. 5C). Surprisingly, the average root hair length in the single nerd1 mutants was significantly longer than that of their wild-type siblings, indicating that NERD1 limits cell growth in this context. As a second surprise, in contrast to the synergistic interaction in primary root growth, exo70A1 nerd1-3 double mutants have short root hairs of similar size to those of exo70A1 single mutants. That is, the effect of the nerd1-3 mutation in increasing average root hair length is masked (epistasis), suggesting that, in root hairs, the exocyst is required for manifestation of NERD1’s root hair length limiting activity.

To determine whether the epistatic interaction of NERD1 and the exocyst was specific, we tested for interactions with another mutation that affects the secretory pathway in root hairs, myosin xi-k. Mutation of myosin xi-k results in shorter root hairs, likely due to inhibition of cytoplasmic streaming that drives secretory vesicle transport (Peremyslov et al., 2008; Peremyslov et al., 2012; Park and Nebenführ, 2013; Peremyslov et al., 2015). The double nerd1-1 myo xi-k mutant demonstrates an additive phenotype: root hairs are longer than with the myo xi-k mutation alone, but not as long as wild-type (Fig. 5D). Thus, the epistatic interaction of nerd1 and exo70A1 mutants is specific, further arguing for a close functional relationship between NERD1 and the exocyst. Moreover, the differing outcomes of the interaction in root hairs versus primary roots...
(epistatic versus synergistic, respectively) argue that this relationship depends on cellular and developmental context.

Additional insight into the role of NERD1 in root hair growth was gained by a closer examination of the morphology of root hairs in nerd1 mutants, which exhibit branches, inflated bases, or bulbous shapes, morphologies that are rare in wild-type siblings (Supplementary Fig. S2). These deviant morphologies are more consistently observed in roots that are growing within agar medium, rather than on the agar surface (where root hairs predominantly extend into the air). Consequently, root hair morphology within the medium was evaluated in 18 roots for each of five genotypes: Col-0 (wild-type), exo84b-1, nerd1-1, nerd1-2, and nerd1-3 (Fig. 6; Supplementary Table S3).

Root hairs in nerd1 mutants, as in wild-type, grow out of the apical end of the trichoblasts at a single location, thus indicating that root hair initiation per se is unaffected in nerd1 mutants. However, nerd1 root hairs are often more bulbous, with wider bases and shanks on average, compared with Col-0 (Fig. 6E). Because nerd1 roots exhibit both wild-type and bulbous root hair morphologies, high standard deviations are associated with nerd1 root hair measurements. Rupture of root hairs, evidenced by the extrusion of cytoplasmic contents into the medium from the root hair tip, is also notable in nerd1 mutants (Fig. 6C and D), occurring in 26–34% of root hairs evaluated, compared with a rare incidence (0–1.8%) in Col-0 or exo84b-1 (Fig. 6F; Supplementary Table S3). Average root hair length for nerd1 root hairs growing within the medium is similar to that in wild-type (nerd1-1: 227 μm; nerd1-3: 247 μm; WT: 230 μm), even though rupture presumably stopped growth in some of the mutant root hairs. Overall, nerd1 root hair morphology is consistent with a role for NERD1 in establishing the structural stability, and perhaps limiting compliance, of the cell wall in growing root hairs. Increased compliance of the cell wall upon loss of NERD1 function might allow for more rapid expansion, leading to increased root hair lengths. But such an effect might also make root hairs vulnerable to bulbous expansion and bursting, as is observed.

Discussion

Secretory events upon which plant growth and development depend are manifested and regulated by a complex network of cellular components interacting both directly and indirectly. Facilitating secretion in many circumstances is an octameric protein complex, the exocyst (Cole and Fowler, 2006; Žárský et al., 2013; Kulich et al., 2015; Vukašinović and Žárský, 2016).

To search for unknown components of the exocyst-mediated secretory network, we used a second-site enhancer screen, and identified nerd1 mutants via their genetic interaction with exocyst mutants, influencing primary root growth, root hair expansion, and hypocotyl elongation in Arabidopsis. Notably, the interaction between NERD1 and the exocyst appears to be indirect, and thus would not have been detected by other methods, e.g. yeast two-hybrid screening. Mutation of NERD1 leads to a shortened root meristem and reduced cell elongation in both the primary root and etiolated hypocotyls. Additionally, mutations of both NERD1 and components of the exocyst affect root hair morphology. The nearly ubiquitous expression of NERD1 throughout the plant (similar to that of most exocyst
components) and its conservation throughout the plant kingdom underline its potential importance in a broader context. Notably, we were unable to generate a doubly homozygous nerd1/exocyst mutant plant from the self-cross of a double heterozygote combining nerd1-3 with severe exocyst mutants (i.e. exo84b-1 and sec8-3; 0 out of 58 and 0 out of 66 individuals genotyped from nerd1/exocyst segregating populations, respectively; P<0.05 by chi-square test for each). This suggests that mutations of NERD1 combined with severe exocyst mutations lead to lethality due to very early developmental defects.

We initially hypothesized that NERD1 directly interacts with the exocyst at the plasma membrane, where exocyst components are known to localize (Fendrych et al., 2010). However, the majority of proteomic studies identify NERD1 in the ER or Golgi (Parsons et al., 2012; Nikolovski et al., 2014; Heard et al., 2015), and not in the plasma membrane (Mitra et al., 2009). Direct examination of the functional NERD1–GFP fusion validated its prominent localization in the Golgi (Fig. 2; Supplementary Fig. S6), in contrast to the preferential association of exocyst components with the plasma membrane (Fig. 3), suggesting that NERD1–exocyst interaction is indirect. Although it remains possible that NERD1 is present at the plasma membrane transiently or at a low level, the most likely interpretation of our results is that the synergistic genetic interaction between NERD1 and exocyst components does not involve direct physical contact.

One alternative hypothesis that does not rely on direct contact to explain the observed NERD1–exocyst genetic interaction is that NERD1 is required for correct exocyst localization; or vice versa, NERD1 is a cargo for exocyst-mediated trafficking. A few specific cargos requiring the plant exocyst for correct delivery have been identified (pectinacious mucilage in Arabidopsis seed coats (Kulich et al., 2010); callose in leaf trichomes (Kulich et al., 2015); and the integral plasma membrane proteins PEN3/ABC36 and NIP5;1 (Mao et al., 2016)). However, no mislocalization of fluorescently tagged exocyst components in nerd1 mutants, or of NERD1–GFP in exocyst mutants, was observed (Fig. 3), arguing against this possibility. On the other hand, these experiments do not exclude genetic interaction via a currently unknown cargo that requires both NERD1 and exocyst-mediated vesicle transport for its proper function.

The localization of NERD1 to the Golgi, the site of synthesis of non-cellulosic polysaccharides incorporated into the cell wall matrix (e.g. pectin and hemicellulose; Driouich et al., 2012; Kim and Brandizzi, 2016), is tantalizing. NERD1 could be involved in the formation or function of trans-Golgi-localized protein complexes, such as the ECHIDNA/YIP4 complex that plays a role in post-Golgi secretion of pectin and hemicellulose to the cell wall, and which also influences cell elongation in roots and hypocotyls (Gendre et al., 2013). However, we currently favor a working hypothesis, based on predicted structure, in which NERD1 directly affects a cell wall matrix polysaccharide, glycoprotein or proteoglycan that is ultimately secreted via exocyst-mediated trafficking to influence cell wall growth and expansion. Most of NERD1 is predicted to be located in the Golgi lumen, folded into a β-propeller-like tertiary structure that could serve as a scaffold for interactions with polysaccharides. Homology modeling suggests that this luminal portion of NERD1 resembles proteins that interact with polysaccharides, i.e. lectins, integrins, and carbohydrate binding proteins. Perhaps most notably, threading programs identify certain NERD1 regions as similar to bacterial RGI pectin lyases, and to a lesser extent xyloglucanases (Supplementary Dataset S2). Interestingly, RGI pectin lyases are activated by calcium, consistent with the calcium-binding pocket predicted for NERD1. Examination of the cell wall in nerd1 roots by, for example, histochemical staining for specific components should help test the hypothesis that NERD1’s impact arises from a role influencing cell wall structure.

It is noteworthy that the phenotypes observed in nerd1 mutants are consistent with cell wall pectins as a target of NERD1 activity. Altering the synthesis of pectic polysaccharides is known to cause a dwarfed phenotype with developmental defects that include shorter primary roots and reduced elongation of etiolated hypocotyls (Reboul et al., 2011), reminiscent of defects observed in nerd1 mutants. RGI pectins impact the same aspects of root hair morphology (e.g. swelling and branching) as those altered in nerd1 mutants (Diet et al., 2006; Reboul et al., 2011). The accelerated cell elongation phase observed in etiolated hypocotyls is the result of cell wall modification, and in particular has been associated with altered pectins (Derbyshire et al., 2007; Pelletier et al., 2010), possibly independent of cellulose synthesis. Thus, a role of NERD1 in the synthesis or modification of cell wall pectins might explain the range of phenotypes observed in nerd1 mutants and deserves further investigation.

Intriguingly, the effect of NERD1 on cell expansion is not uniform throughout development: nerd1 mutations result in reduced cell elongation in the root tip and etiolated hypocotyl, but increased elongation of root hairs, along with increased likelihood of rupture at the growing root hair tip. It is also of note that a mutation altering Arabidopsis EXO70C2, another potential indirect exocyst interactor, leads to a similar phenotype in the tip-growing pollen tube: more rapid growth and increased tube rupture (Synek et al., 2017). The contrasting effects of nerd1 mutants on root hair tip growth versus primary root cell elongation could manifest because the cell wall matrices in the two cell types are structurally distinct from each other, generated by fundamentally different processes: delivery of non-cellulosic cell wall components to a narrowly focused region versus more broadly distributed modification of a pre-existing cell wall matrix, respectively. Additional indirect evidence that cell walls are differentially altered is the increased incidence of bulging in nerd1 root hair shafts, although neither bulging nor rupture is characteristic of cells in mutant root meristematic and elongation zones. In the root, the composition and structure of the cell wall changes as cells progress through division, elongation, and differentiation zones. For example, the rhamnogalacturonan I pectin in cell walls is modified during the transition from cell proliferation to cell elongation in roots (Willats et al., 1999), and pectins in root hairs are structurally distinct from those in the lateral cell walls elsewhere in the primary root (Muszyński et al., 2015). Thus, the composition of cell wall components available for interaction with NERD1 within the Golgi likely varies as root cells progress.
from elongation to differentiation. Such differences could alter NERD1’s impact on cell wall extensibility, elongation, and fragility in a developmentally dependent manner. Revealing the molecular function of NERD1 should help define how it is integrated with secretory system to help determine developmentally stage-specific patterns of cell growth and expansion.

Supplementary data
Supplementary data are available at JXB online.

Methods. In silico prediction of NERD1 tertiary structure, with references.

Dataset S1. Alignment of NERD1 homologs.
Dataset S2. Threading analysis of NERD1.
Dataset S3. Alignment of NERD1 homologs, Jalview format.

Fig. S1. SHOR-Emap identification of nerd1 candidate genes.
Fig. S2. Images of root hair morphology in nerd1.
Fig. S3. RT-PCR for nerd1 mutants.
Fig. S4. Diagram of predicted domains in NERD1 protein.
Fig. S5. Model depicting predicted tertiary structure of NERD1.
Fig. S6. Localization of NERD1–GFP to Golgi using NAG::Turq.
Fig. S7. Localization of NERD1–GFP changes in response to BFA.
Fig. S8. NERD1 acts synergistically with SEC8 to affect hypocotyl elongation.
Table S1. List of primers used for PCR and RT-PCR.
Table S2. Domains within NERD1 protein.
Table S3. Morphology of nerd1 root hairs.

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