RESEARCH PAPER

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 intercellular 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; Synek *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, Ral, 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 (Synek *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 autophagy (Kulich *et al.*, 2013), Exo70I 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; Synek *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): *nerd1-2* (At3g51050, SALK 018060C); *nerd1-3* (At3g51050, SALK 051660); *exo70A1-2* (At5g03540, SALK 135462); *sec8-3* (At3g10380, SALK 026204); *sec8-4* (At3g10380, SALK 118129); *sec8-6* (At3g 10380, SALK 091118); and *myo XI-K* (At5g20490, SALK 067972). The *exo84b-1* line was a GABI-Kat line (Rosso *et al.*, 2003; Fendrych *et al.*, 2010). The *EXO84b-GFP* and *GFP-SEC8* lines were previously described (Fendrych *et al.*, 2010). The *nerd1-1* 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 228C 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 Endosidin2, three groups were germinated on MS plates: seedlings that were homozygous for *nerd1-2*; *nerd1-2* 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 Endosidin2 (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 *nerd1-1* 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. SHOREmap 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 *Ava*II 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 *Sbf*I and *Pac*I sites. Enhanced GFP (EGFP; 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 *STtmd-YFP* or *NAG-mTurq* (Peremyslov *et al.*, 2012) at concentrations equal to 0.2 OD_{600}. 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-Apochromat ×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 endomembrane 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 system. Multiple digital images were taken to capture two cortical cell files for each root, one on each side of the longitudinal midline from the quiescent center to the differentiation zone. Cell widths and lengths were measured. The cortical cell length profile combined with the root growth rate allowed estimations of the number of cells in the meristem, the cell production rate, and the length of the cell cycle for each root. 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 SV 11 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 dwarfism 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 (Synek *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
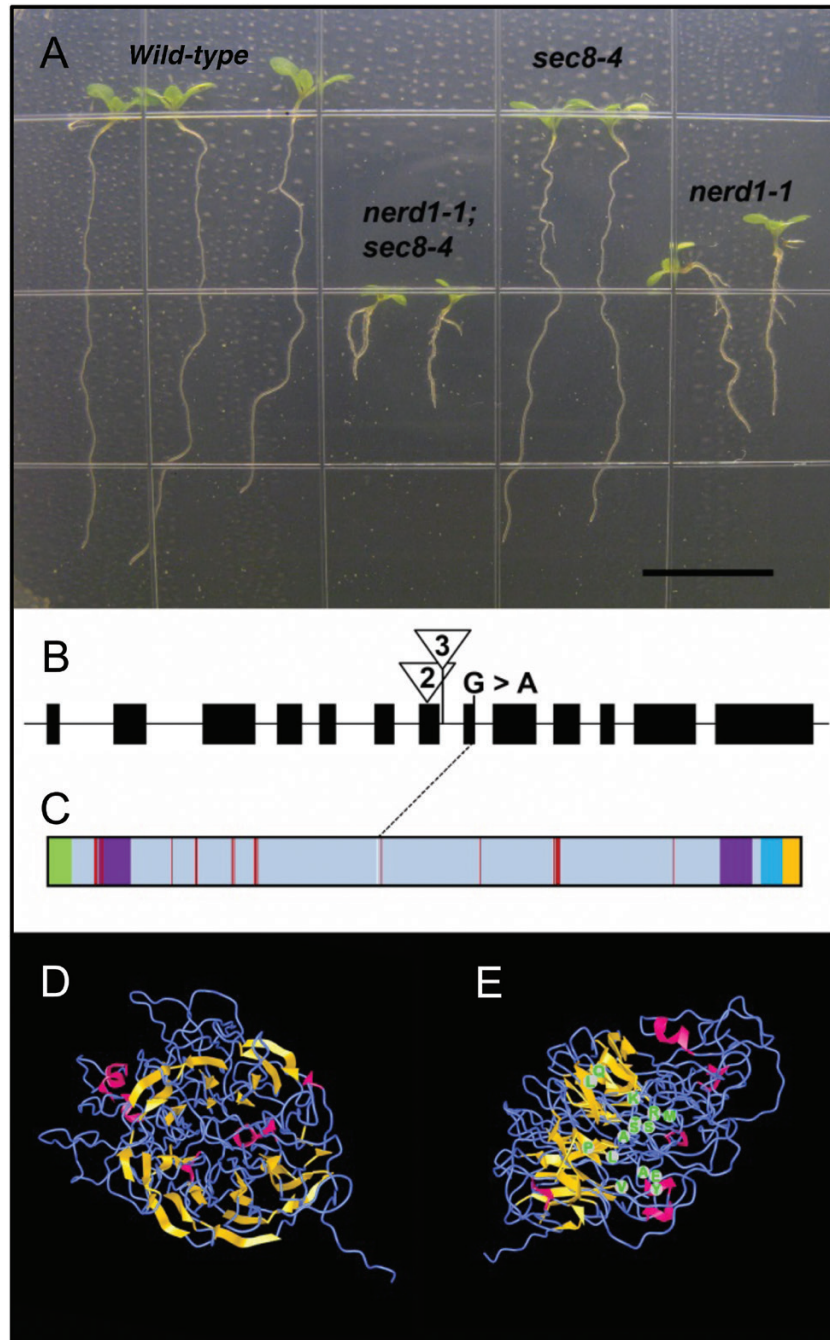
Fig. 1. *NERD1* encodes a transmembrane domain protein important for wild-type root development. (A) The combination of *nerd1-1* and *sec8-4* mutations results in a more severe root growth defect than either single mutation. Scale bar: 1 cm. (B) Map showing exons of *NERD1* (At3g51050), the site of the G→A point mutation at a splice junction in *nerd1-1*, and the sites of the T-DNA insertions *nerd1-2* (triangle 2: SALK_018060) and *nerd1-3* (triangle 3: SALK_051660). (C) Schematic representation of NERD1 primary sequence features, showing an N-terminal signaling peptide (green), FG–GAP domains (purple), transmembrane domain (aqua), cytoplasmic domain (yellow), and residues predicted to form a calcium-binding pocket by RaptorX (red). The blue diagonal line between (B) and (C) shows where the splice site mutation in *nerd1-1* is predicted to affect the NERD1 polypeptide. (D) NERD1 tertiary structure predicted by RaptorX, showing a β-propeller in yellow and α-helices in magenta. (E) Side view of the model in (D) with locations of residues in the predicted calcium-binding pocket (green).

segregation pattern further shows that *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), asso-ciated with lowest expression of At3g51050. The absence of a significant transmission defect in *nerd1* mutants, and the low level of expression, suggests that *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 prod-uce non-functional protein. NERD1 transcripts in *nerd1-1*

homozygotes 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 EnsemblPlants 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 26 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 (Supplmentary Dataset S1).

Protein modeling software was used to predict potential structural characteristics of NERD1 (Fig. 1C; Supplementary Table S2; Supplementary Figs S4 and S5; Supplmentary Datasets S2 and S3). Arabidopsis NERD1 contains an N-terminal signaling peptide that is well conserved across all plant species and is indicative of association with the secretory system (TargetP 1.1, Emanuelsson *et al.*, 2000). A single-pass transmembrane domain is identified near the C-terminus of the protein, leaving a short 18–23 amino acid cytoplasmic tail. Homology threading programs (3DLigandSite, Wass *et al.*, 2010; Raptor-X, Källberg *et al.*, 2012; SWISS-Model, Biasini *et al.*, 2014; Phyre2, Kelley *et al.*, 2015) that compared NERD1's primary and secondary structure with proteins with tertiary structures solved by x-ray crystallography (Supplmentary Dataset S2) predict (with 90–98% confidence) that the non-cytoplasmic portion of NERD1 folds into a globular protein with a β-propeller structure: seven predicted β-sheets arranged radially and pseudosymmetrically 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 (Supplmentary 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 *Nicotiana 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*-acetylglucosaminyl 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
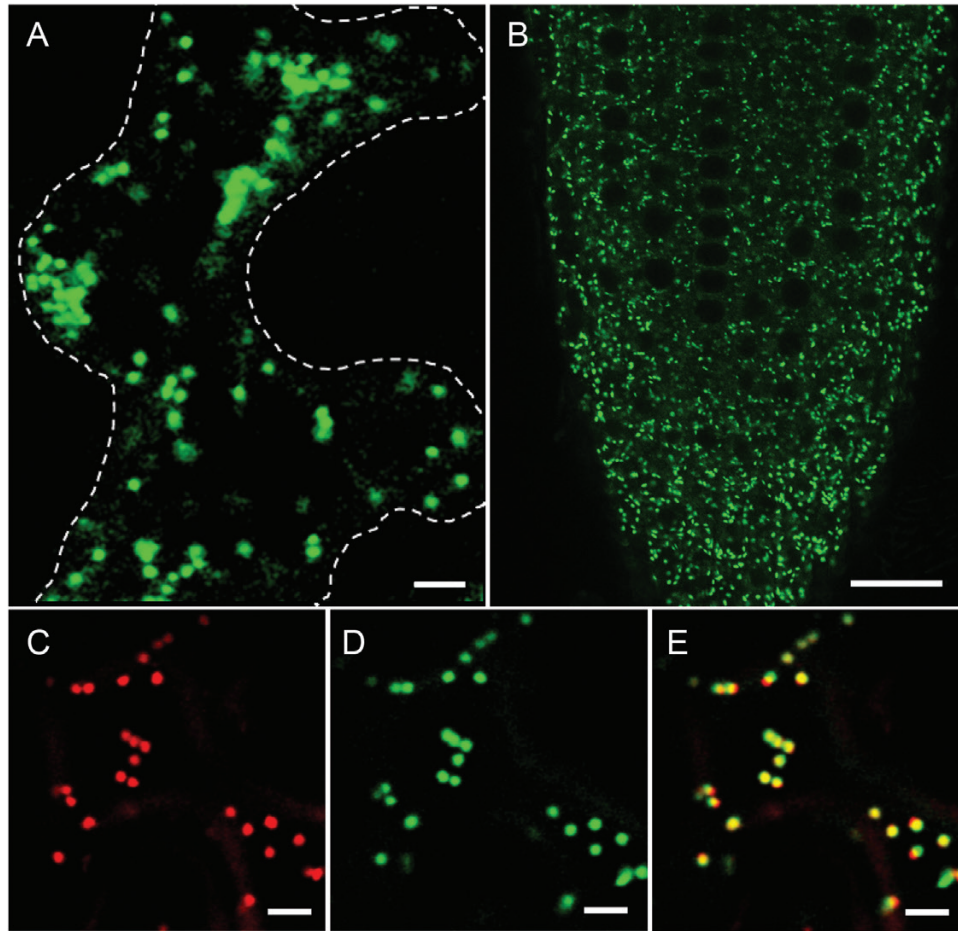
Fig. 2. NERD1–GFP localizes to the Golgi. (A) NERD1–GFP in *Nicotiana benthamiana* leaf cells. (B) Root tip of a *NERD1-GFP*-complemented *nerd1-2* mutant. (C–E) Co-localization of NERD1 with the Golgi marker: (C) STtmd::mCherry (sialyltransferase transmembrane domain), (D) NERD1–GFP, and (E) merged image. Scale bars: 5 μm (A, C, D, E) and 20 μm (B).

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 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
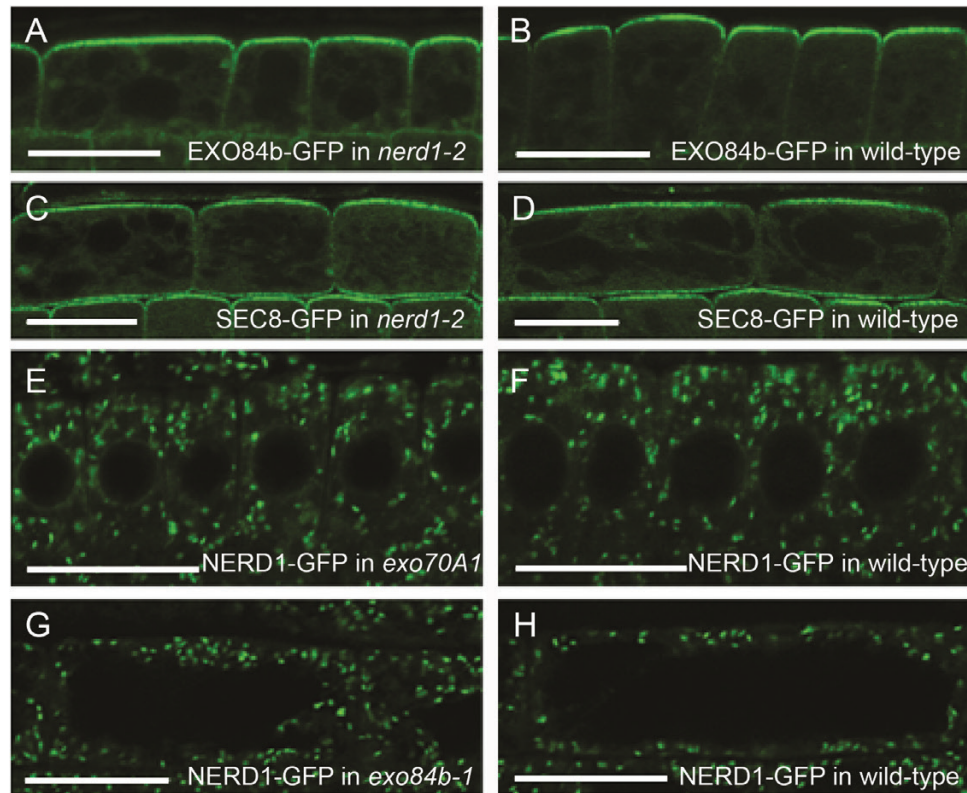
Fig. 3. Subcellular localizations of NERD1 and exocyst markers are independent of each other. The localization of exocyst markers to the outer surface of root epidermal cells of *nerd1-2* mutants (A, C) is similar to that in wild-type siblings (B, D). Conversely, the predominant localization of NERD1–GFP 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.)

function by interacting with EXO70A1 (Zhang *et al.*, 2016). 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.
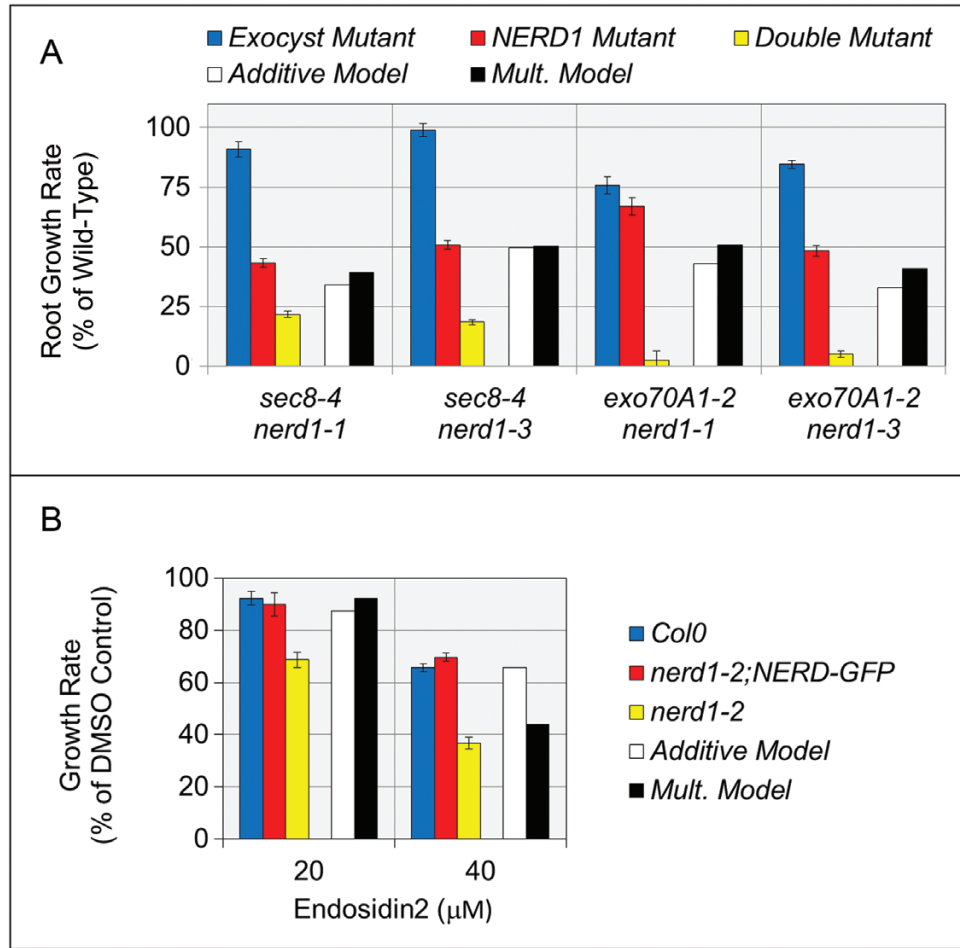
Fig. 4. NERD1 acts synergistically with exocyst components to affect primary root growth. (A) Mutation of exocyst components *sec8-4* or *exo70A1-2* results in a mild reduction in primary root growth rate compared with wild-type (blue bars), while mutation of *nerd1* (*nerd1-1* or *nerd1-3*, red bars) results in an approximately 50% reduction in growth rate. The combination of a mutation in an exocyst component with a *nerd1* mutation (green bars) results in a severe root growth defect that is more severe than predicted by additive ($P<0.001$, *z*-test) or multiplicative models ($P<0.0001$, *z*-test), indicating a synergistic interaction. (Error bars: SE; n=19–67 roots for each genotype.) (B) Compared with DMSO-treated controls, root growth was significantly more reduced in *nerd1-2* mutants when 20 or 40 µM Endosidin2, an exocyst inhibitor, was added to the growth medium, compared with either Col-0 or *nerd1-2; NERD-GFP* complemented seedlings. At both concentrations, the reduced growth rate in the *nerd1-2* mutant treated with Endosidin2 was more than would be predicted by additive or multiplicative models ($P<0.001$, *z*-test). (Error bars: SE; n=13–17 roots for each genotype/Endosidin2 concentration.)

Table 1. *Primary root growth parameters of* nerd1 *mutants compared with Col-0 and* exo84b-1

Genotype	Roots evaluated	Root growth (µm h⁻¹)		Meristem size (no. of cells)		Mature cell length (µm)			Mature cell width (µm)			Cell production (cells h⁻¹)		Estimated length of cell cycle (h)	
		Mean	SD	Mean	SD	n^a	Mean	SD	n^a	Mean	SD	Mean	SD	Mean	SD
Col-0	9	469.3	43.8	49.6	4.4	264	200.9	43.0	84	28.0	2.57	2.33	0.24	14.7	1.5
exo84b-1	10	51.2	6.6	10.0	1.9	278	86.6	21.8	84	13.2	1.53	0.59	0.08	11.4	1.1
nerd1-1	6	**248.1**	30.9	**33.8**	4.0	82	**144.0**	30.1	89	*27.0*	3.98	**1.77**	0.23	13.3	1.5
nerd1-2	6	**231.2**	38.8	**32.4**	3.0	112	**153.3**	38.5	87	*28.4*	3.34	**1.50**	0.17	*14.5*	0.8
nerd1-2	6	**262.7**	21.6	**35.3**	0.9	89	**130.7**	36.5	75	**29.9**	3.81	*2.08*	0.41	12.0	2.3

Values highlighted in bold are significantly different from Col-0 and *exo84b-1* ($P<0.001$, *t*-test). Values highlighted in italic are significantly different from *exo84b-1*, but not Col-0 ($P<0.001$, *t*-test).
[a] n=number of cells measured.

Polarized growth of root hairs

One phenotype leading to selection of the initial *nerd1* allele was altered root hair morphology. Short root hairs are charac–teristic 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).
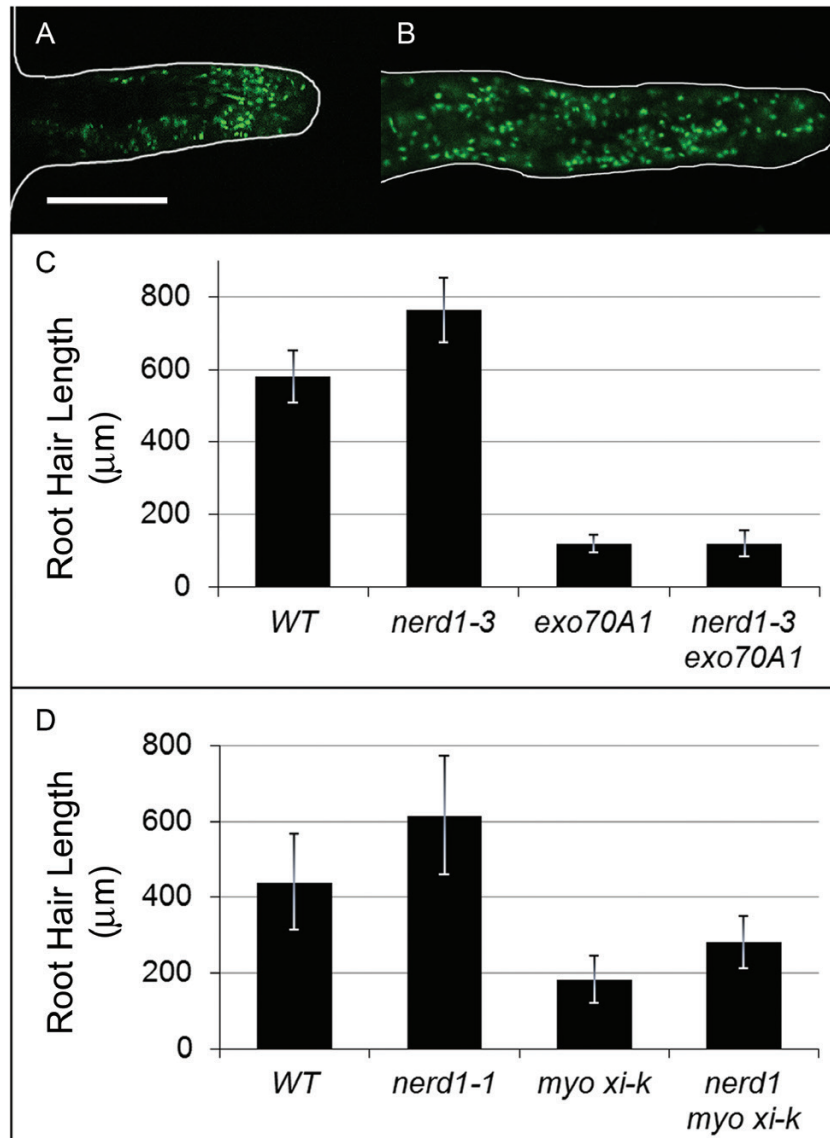
Fig. 5. NERD1 is epistatic to the exocyst in the root hair. Root hair lengths were examined for plants growing on vertical plates so that their roots were on the surface of the growth medium, i.e. at the medium–air interface. NERD1–GFP is localized to mobile punctate structures in both wild-type (A) and *exo70A1* (B) root hairs (scale bars for A and B: 20 µm). Mature root hairs of *nerd1-3* and *nerd1-1* mutants are longer than for wild-type siblings (*P*<0.0001, *t*-test). (C) Extremely short root hair lengths are observed in *exo70A1* mutants compared with wild-type (*P*<0.0001), and this phenotype is not altered by the addition of a *nerd1-3* mutation in the double mutant (*P*=0.3). (*n*=190 for WT, *nerd1-3*, and *exo70A1*, *n* =51 for double mutant.) (D) The *myo xi-k* mutant has short root hairs compared with wild-type (*P*<0.0001). In *nerd1-1; myo xi-k* double mutants, root hairs are longer than those of *myo xi-k* (*P*<<0.0001), but still far shorter than wild-type (*P*<0.001). (*n*>164 for WT, *nerd1-1* and *myo xi-k*; *n*=79 for double mutant; error bars: SD.) (This figure is available in colour at *JXB* online.)

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
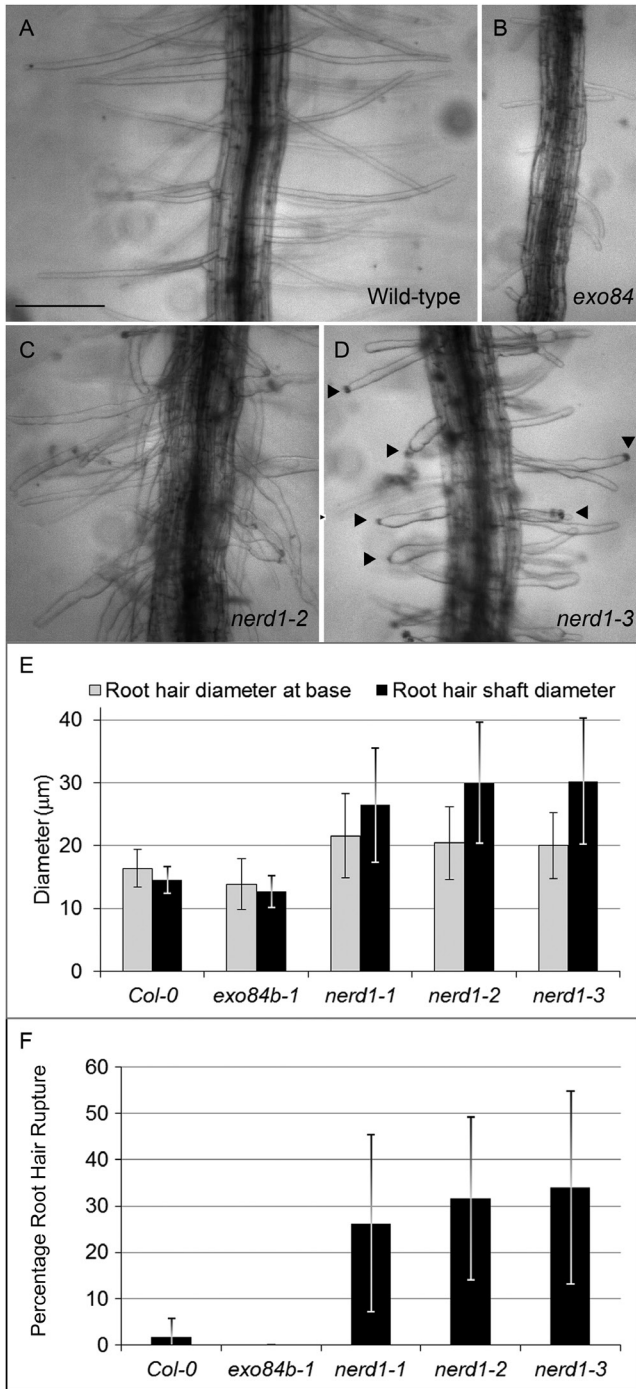
Fig. 6. NERD1 affects morphology of root hairs growing within agar medium. (A) Col-0 (wild-type), (B) *exo84b-1*, (C) *nerd1-2*, (D) *nerd1-3*. Arrowheads: ruptured root hair tips; scale bar: 200 µm. (E, F) Root hairs of *nerd1* mutants (*n*=~200 root hairs for each allele) were significantly wider at the base and at the widest part of their shaft than either Col-0 (*n*=203) or *exo84b-1* (*n*=79) (E), and showed a significantly higher incidence of root hair rupture (F). (Root hairs measured from 18 roots for each genotype, *P*<0.001, *t*-test; error bars: SD.)

(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. These defects are also seen in plants with mutations affecting exocyst components, but are synergistically accentuated when both *nerd1* and exocyst mutations are combined. 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 king-dom 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 het-erozygote 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 *nerd*/exocyst segregating populations, respect-ively; *P*<0.05 by chi-square test for each). This suggests that mutations of *NERD1* combined with severe exocyst muta-tions 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 indir-ect. 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/ABCG36 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 syn-thesis of non-cellulose 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-local-ized protein complexes, such as the ECHIDNA/YIP4 complex that plays a role in post-Golgi secretion of pectin and hemi-cellulose to the cell wall, and which also influences cell elong-ation 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 ter-tiary structure that could serve as a scaffold for interactions with

polysaccharides. Homology modeling suggests that this lume-nal portion of NERD1 resembles proteins that interact with polysaccharides, i.e. lectins, integrins, and carbohydrate bind-ing proteins. Perhaps most notably, threading programs iden-tify 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 polysaccha-rides is known to cause a dwarfed phenotype with develop-mental defects that include shorter primary roots and reduced elongation of etiolated hypocotyls (Reboul *et al.*, 2011), rem-iniscent 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 pheno-type 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 pri-mary 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 evi-dence 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 compos-ition 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 mod-ified during the transition from cell proliferation to cell elong-ation 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 com-position 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.

Acknowledgements

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