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ORIGINAL ARTICLE

Characterization of in vivo functions of *Nicotiana benthamiana* RabE1

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Abstract We characterized the gene expression, subcellular localization, and in vivo functions of a *Nicotiana benthamiana* small GTPase belonging to the RabE family, designated NbRabE1. The *NbRabE1* promoter drove strong *β*-glucuronidase reporter expression in young tissues containing actively dividing cells and in stomata guard cells. GFP fusion proteins of NbRabE1 and its dominant-negative and constitutively active mutants were all localized to the Golgi apparatus and the plasma membrane but showed different affinities for membrane attachment. Virus-induced gene silencing of *NbRabE1* resulted in pleiotropic phenotypes, including growth arrest, premature senescence, and abnormal leaf development. At the cellular level, the leaves in which *NbRabE1* was silenced contained abnormal stomata that lacked pores or contained incomplete ventral walls, suggesting that NbRabE1 deficiency leads to defective guard cell cytokinesis. Ectopic expression of the dominant-negative mutant of NbRabE1 in *Arabidopsis thaliana* resulted in retardation of shoot and root growth accompanied by defective root hair formation. These developmental defects are discussed in conjunction with proposed functions of RabE GTPases in polarized secretory vesicle trafficking.

Keywords Growth retardation · Guard cell division · Promoter–GUS fusion · Root hair growth · Subcellular localization · Virus-induced gene silencing

C.S. Ahn and J.-A. Han contributed equally to this work.

Genbank accession number: JQ519194 (NbRabE1).

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Introduction

Small GTP-binding proteins regulate diverse processes in eukaryotic cells, such as signal transduction, cell proliferation, cytoskeletal organization, and intracellular membrane trafficking (Boguski and McCormick 1993; Takai et al. 2001). The *Arabidopsis thaliana* genome contains 93 genes that encode small GTP-binding proteins, among which 57 members belong to the Rab family (Vernoud et al. 2003). The Rab family GTPases are typically localized to the cytosolic face of specific intracellular membranes, where they function as regulators of distinct steps in membrane trafficking pathways (Zerial and McBride 2001; Stenmark and Olkkonen 2001). Among these, five *Arabidopsis* RabE isoforms share significant homology with yeast and mammalian Rab GTPase isoforms that regulate polarized secretion from the *trans*-Golgi network (TGN) to the plasma membrane (Vernoud et al. 2003). The yeast isoform Sec4p regulates polarized vesicle transport from the Golgi apparatus to daughter cell bud sites (Salminen and Novick 1987; Goud et al. 1988). Sec4p is involved in formation of the exocyst complex and association of the complex with secretory vesicles to target them to the plasma membrane (Zerial and McBride 2001; Ponnambalam and Baldwin 2003). The mammalian isoform Rab8 is functionally associated with the membrane traffic route from the TGN to the plasma membrane for protrusion formation and apical protein localization by targeting the mammalian exocyst to a subset of secretory vesicles (Huber et al. 1993; Ponnambalam and Baldwin 2003; Hattula et al. 2006; Sato et al. 2007). In plants, polarized membrane trafficking from the Golgi apparatus to the plasma membrane is required for cell plate formation, cell expansion, and asymmetric localization of membrane proteins and lipids, suggesting possible involvement of plant RabE proteins in those processes (Carter et al. 2004).

Mechanisms of RabE-mediated membrane trafficking in plant cells were studied using a transient trafficking assay in tobacco leaf epidermis (Zheng et al. 2005). A dominant-negative (DN) mutant of RabE1d was found to function downstream of the Rab-D subclass and inhibit membrane trafficking from the Golgi apparatus to the plasma membrane (Zheng et al. 2005). To suggest a physiological function of plant RabE proteins, a tomato RabE family GTPase was identified to interact with avrPto, an effector protein produced by the plant pathogen *Pseudomonas syringae*, only in the absence of the resistance protein Pto (Bogdanove and Martin 2000). This finding suggests that avrPto may interfere with RabE-regulated polarized secretion of antimicrobial compounds and defense proteins in susceptible plants. Consistent with this result, overexpression of a constitutively active (CA) mutant form of one of the five *Arabidopsis* RabE proteins, RabE1d-Q74L, conferred resistance to *Pseudomonas syringae* on the transgenic *Arabidopsis* plants via enhanced plant defense responses (Speth et al. 2009). RabE1d and related GTPases also seem to play a critical role in plant growth and development, because cosuppression using the *RabE1d* sequence resulted in significantly reduced plant size and abnormal leaf development in *Arabidopsis* (Speth et al. 2009). In this study, to explore diverse functions of other RabE isoforms, we investigated the distribution and in vivo functions of *N. benthamiana* RabE1.

Materials and methods

Virus-induced gene silencing (VIGS)

Various *NbRabE1* cDNA fragments were PCR-amplified and cloned into a pTV00 vector containing part of the TRV genome. VIGS was performed as described (Kim et al. 2006; Lee et al. 2009; Ahn et al. 2011). The fourth leaf above the infiltrated leaf was used for real-time quantitative RT-PCR.

GUS histochemical assay

The 1,979-bp *NbRabE1* promoter was isolated from *N. benthamiana* genomic DNA using the Genome Walker™ kit (Clontech). The isolated promoter was cloned into the pBI101 vector to generate an in-frame *NbRabE1* promoter–*GUS* fusion gene. The recombinant Ti-plasmid was introduced into *Agrobacterium tumefaciens* LBA4404, and the *Agrobacterium* strain was used to transform *Arabidopsis* plants. Fixation, staining, and clearing of the transgenic *Arabidopsis* seedlings with X-Gluc was performed as described previously (Jefferson et al. 1987).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was carried out to detect the endogenous *NbRabE1* transcript using the primers (5′-cagaaaagccaagcaaaagc-3′ and 5′-cacgttaaatttcaaaagaatg acc-3′) corresponding to the 3′-UTR of the *NbRabE1* mRNA as described (Ahn et al. 2011).

Generation of dominant-negative and constitutively active mutant forms of NbRabE1

Overlapping PCR was carried out to generate point mutations for the amino-acid substitutions Ser-29 to Asn (S29N; DN) and Gln-74 to Leu (Q74L; CA) in the *NbRabE1* cDNA in the pBluescript plasmid as described (Speth et al. 2009). The primers used to introduce the mutation were 5′-acagc tggtctggaacgg-3′ and 5′-ccgttccagaccagctgt-3′ for the CA mutant and 5′-ggtgttggtaagaattgccttc-3′ and 5′-gaaggcaatt cttaccaacacc-3′ for the DN mutant. The primers 5′-ggatccg atggcagcacc-3′ and 5′-ggatcccgaaccacagcaa-3′ were used to amplify the mutant *NbRabE1* genes for cloning.

Subcellular localization of NbRabE1 and its mutants

The cDNAs corresponding to the wild-type, dominant-negative, and constitutively active forms of NbRabE1 were cloned into the SmaI and BglII sites of the sGFP-pCAMBIA1390 plasmid to generate GFP:NbRabE1, GFP:NbRabE1(DN), and GFP:NbRabE1(CA) fusion proteins, respectively. The GFP fusion proteins, alone or in combination with a Golgi marker G-RK (Nelson et al. 2007), were transiently expressed in leaves of 3-week-old *N. benthamiana* plants by agroinfiltration as described (Voinnet et al. 2003). After 48 h, protoplasts were generated, and the GFP signal was detected by confocal laser scanning microscopy (Carl Zeiss LSM 510) as described previously (Cho et al. 2004). For visualization of the plasma membrane, the protoplasts were stained with FM4-64 (Invitrogen Molecular Probes) at a final concentration of 8.2 μM for 1 min at room temperature before observation. Co-localization percentages of green fluorescent signals of the GFP fusion proteins and red fluorescence of FM4-64 were determined by the ImageJ program.

Immunoblot analyses

The GFP:NbRabE1, GFP:NbRabE1(DN), and GFP:NbRabE1(CA) fusion proteins, together with the membrane marker PM-CK (Nelson et al. 2007), were transiently expressed in leaves of 3-week-old *N. benthamiana* plants by agroinfiltration. Protein extracts (30 μg) extracted from the infiltrated leaves were separated by SDS-PAGE and transferred to Immobilon-P membranes in transfer buffer (10 mM Tris, 192 mM glycine, and 20 % methanol).

Membrane preparation and western blotting were performed according to the manufacturer's instructions using monoclonal mouse antibodies against the GFP tag (1:10,000 dilution; ABM) and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:10,000 dilution; Invitrogen). Signals were detected using an enhanced chemiluminescence kit (GE Healthcare). To detect HA-fused forms of NbRabE1 in transgenic *Arabidopsis* plants, immunoblotting was performed as described above using anti-HA antibodies (1:10,000 dilution; ABM).

DAPI staining

DAPI staining and detection by confocal laser scanning microscopy was performed as described (Cho et al. 2004).

Generation of *Arabidopsis* transgenic lines that overexpress wild-type or mutant NbRabE1

The HA-fused NbRabE1, NbRabE1(CA), and NbRabE1 (DN) constructs under the control of the CaMV35S promoter were cloned into the binary vector pCAMBIA1390. The recombinant plasmid containing each construct was introduced into *Agrobacterium tumefaciens* (C58C1) and transformed into *Arabidopsis* using *Agrobacterium*-mediated transformation. Transgenic plants were selected on growth medium containing hygromycin (30 mg/L). To examine seedling growth, seeds were surface-sterilized and plated on Murashige and Skoog salts (MS) medium (GIBCO-BRL) solidified with 0.6 % Phytagel (Sigma).

Results

Identification of *N. benthamiana* RabE1

We have carried out functional genomics using tobacco rattle virus (TRV)-based VIGS in *N. benthamiana* to assess functions of various signal transduction genes (Kim et al. 2006; Lee et al. 2009; Ahn et al. 2011). The screening revealed that gene silencing of a member of the RabE GTPase family, designated *NbRabE1*, caused severe developmental phenotypes in *N. benthamiana*. The full-length *NbRabE1* cDNA encodes a polypeptide of 216 amino acids, corresponding to a molecular mass of 23,760 Da. A phylogenetic tree of NbRabE1 and the five *Arabidopsis* RabE family proteins indicates that NbRabE1 is grouped with *Arabidopsis* RabE1a, RabE1b, and RabE1c (Fig. 1a).

Expression patterns of the *NbRabE1* promoter–*GUS* fusion gene in transgenic *Arabidopsis* plants

To examine the expression pattern of *NbRabE1*, we generated transgenic *Arabidopsis* plants carrying the *NbRabE1* promoter–*GUS* fusion gene that consists of a 1,979-bp *NbRabE1* promoter fused in-frame to the *GUS* gene. Based on histochemical GUS staining, *NbRabE1* promoter activity was strongly detected in young tissues containing dividing cells, such as the shoot apex, young leaves, developing vasculature, root tips, lateral roots and stomata in the transgenic plants (Fig. 1b A–I). During stomata development, GUS staining was strongly observed in young guard cells and the staining became weaker as the guard cells further developed, while only a small percentage of guard mother cells, presumably just prior to cell division, exhibited GUS staining (Fig. 1b J–N). GUS staining was not observed in meristemoids (results not shown).

Subcellular localization of wild-type and mutant NbRabE1

Rab GTPases are present in a cell in two forms: a cytosolic form and a membrane-associated form, depending on GTP/GDP binding state and interaction with accessory proteins (Zerial and McBride 2001). Particularly, specific localization of Rab proteins to the target membrane is critical for their function (Zerial and McBride 2001; Stenmark and Olkkonen 2001). NbRabE1 is grouped with RabE1a, RabE1b, and RabE1c of the five RabE proteins in *Arabidopsis* (Fig. 1a). To identify the target membrane of NbRabE1, GFP fusion proteins of NbRabE1, NbRabE1(DN), a dominant-negative mutant containing the Ser-29 to Asn mutation (S29N), and NbRabE1(CA), a constitutively active mutant containing the Gln-74 to Leu mutation (Q74L), were transiently expressed in *N. benthamiana* leaves by agroinfiltration (Fig. 2). After 48 h, expression of GFP fusion proteins in protoplasts generated from the infiltrated leaves was examined by confocal laser scanning microscopy (Fig. 2a, b). To visualize the plasma membrane, the protoplasts were briefly stained with FM4-64, a lipophilic dye that is rapidly incorporated into the plasma membrane (Bolte et al. 2004). To visualize the Golgi complex, the Golgi marker protein G-RK was co-expressed by agroinfiltration (Nelson et al. 2007). Optical sections of the green fluorescent signal suggested that NbRabE1 and its mutants are mainly associated with the plasma membrane and the Golgi apparatus, suggesting that these are the target membranes of the NbRabE1 GTPase (Fig. 2a, b). Furthermore, co-localization percentages of green fluorescent signals of the GFP fusion proteins and red fluorescence of FM4-64 indicate that GFP:NbRabE1(CA) is most closely associated with the plasma membrane, followed by GFP:NbRabE1 and GFP:NbRabE1(DN) (Fig. 2a).

In addition, soluble and membrane protein fractions were purified from the agroinfiltrated *N. benthamiana* leaves and subjected to immunoblot analyses using anti-GFP

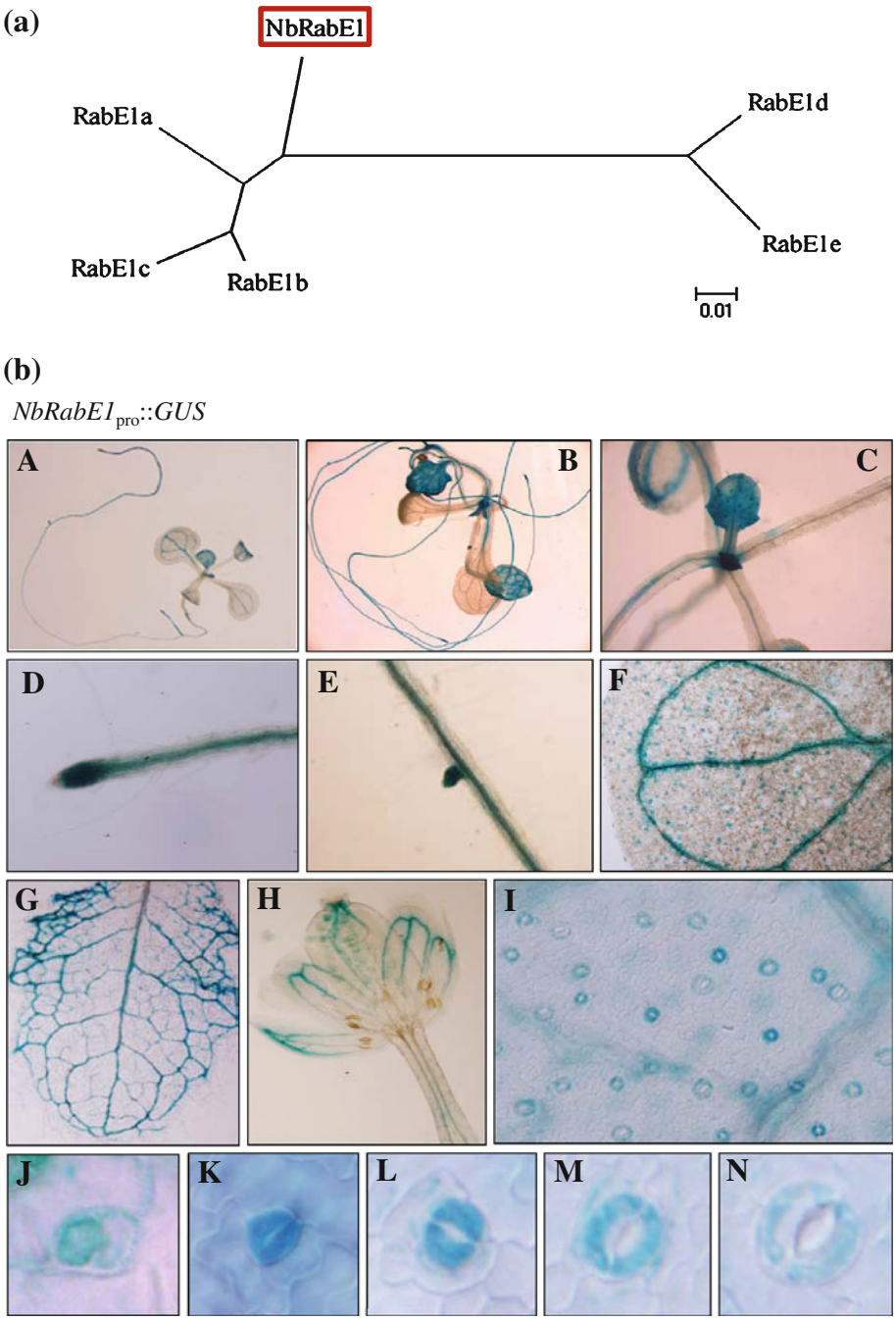

Fig. 1 Phylogenetic relationship and histochemical GUS staining. **a** Phylogenetic tree of *NbRabE1* and five *Arabidopsis RabE* family genes was drawn by the neighbor-joining bootstrap method using Mega 4.1 based on multiple alignments of RabE amino-acid sequences using ClustalX 1.81. The *scale bar* represents 0.01 amino-acid substitutions per site in the primary structure. **b** In *Arabidopsis* transgenic plants carrying the *NbRabE1* promoter–*GUS* fusion gene, GUS staining is shown in the following tissues: whole seedling at 5 days after sowing (DAS) and (*B*) at 8 DAS, (*C*) the shoot apex and young leaves at 5 DAS, (*D*) the root tip and root vasculature, (*E*) lateral roots, (*F*) vasculature and stomata of cotyledon, (*G*) young leaves, (*H*) vascular tissues in flowers, (*I*) stomata in leaves, (*J*) guard mother cells, and (*K–N*) guard cells in different developmental stages

antibodies (Fig. 2c). As a membrane marker, plasma membrane-localized PM-CK was co-expressed with NbRabE1 and its mutant proteins (Nelson et al. 2007). Based on the immunoblotting results, GFP:NbRabE1(CA) was mostly associated with the membrane compartment and GFP:NbRabE1(DN) was present mainly in the cytosol, while GFP:NbRabE1 was present in both fractions, indicating that NbRabE1 activation leads to its association with the target membrane. *Arabidopsis* RabE1d had also previously been found to associate with the Golgi apparatus and the plasma membrane in *Arabidopsis* leaf cells (Speth et al. 2009). These results are consistent with the findings that the yeast and mammalian RabE homologs regulate membrane traffic from the TGN to the plasma membrane (Zerial and McBride 2001; Ponnambalam and Baldwin 2003).

VIGS of *NbRabE1*

Physiological functions of *NbRabE1* were examined using VIGS in *N. benthamiana*. We cloned three different

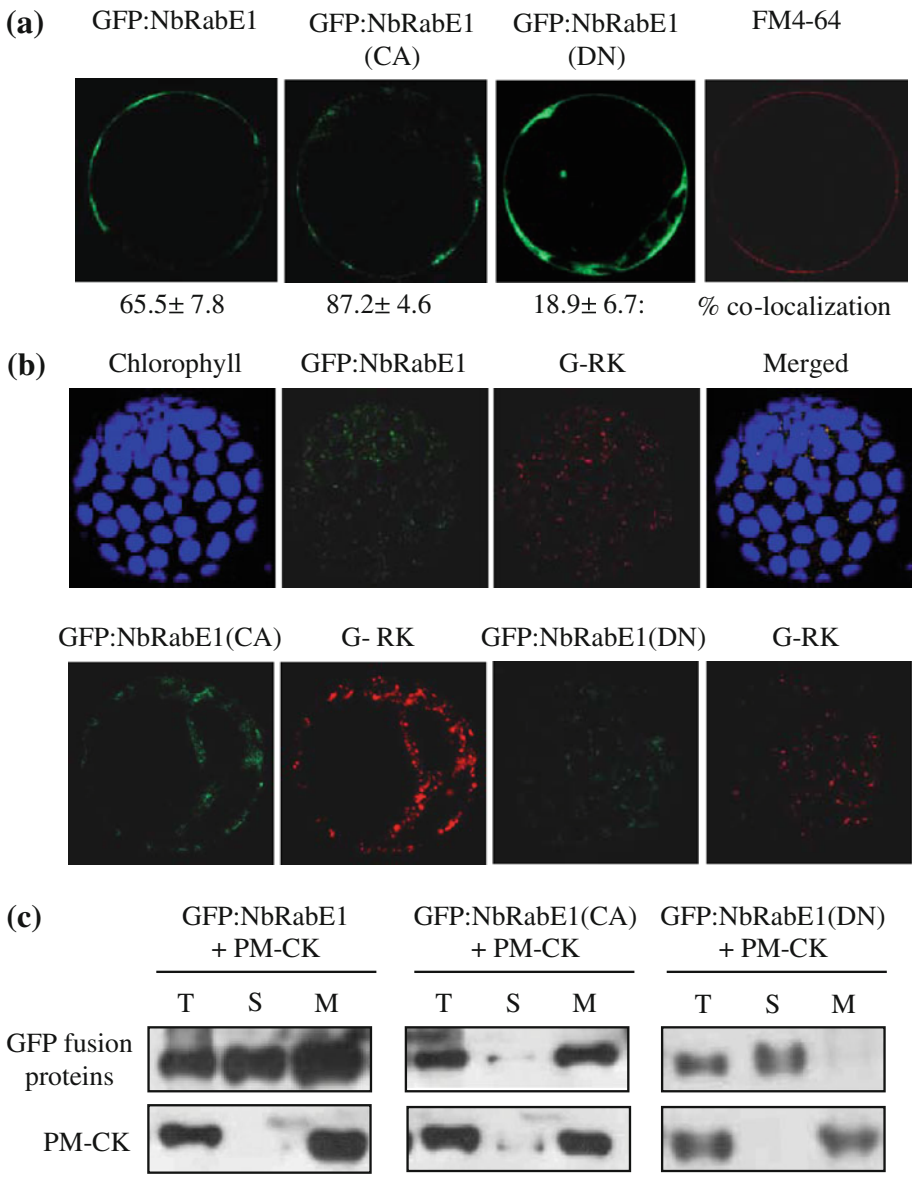

Fig. 2 Subcellular localization of wild-type and mutant NbRabE1. **a** GFP:NbRabE1, GFP:NbRabE1(CA), and GFP:NbRabE1(DN) fusion proteins were expressed in *N. benthamiana* leaves through agroinfiltration. Protoplasts isolated from the infiltrated leaves were examined by confocal laser scanning microscopy. To mark the plasma membrane, the protoplasts were briefly stained with FM4-64 dye before examination. Optical sections revealed green fluorescent signals of the GFP fusion proteins in the plasma membrane. Co-localization percentages of green fluorescent signals of the GFP fusion proteins and red fluorescence of FM4-64 are presented below the confocal microscope images. **b** To mark the Golgi complex, G-RK was co-expressed with the GFP:NbRabE1 fusion proteins. Green fluorescent signals of the GFP fusion proteins overlapped with red fluorescence of G-RK, suggesting that wild-type and mutant NbRabE1 proteins are associated with the Golgi apparatus. **c** GFP:NbRabE1, GFP:NbRabE1(CA), and GFP:NbRabE1(DN) fusion proteins were expressed together with a membrane marker protein PM-CK through agroinfiltration. Protein extracts prepared from the infiltrated leaves were analyzed by immunoblotting with anti-GFP antibody. *T* total fraction, *S* soluble fraction, *M* membrane fraction

fragments of the *NbRabE1* cDNA into the VIGS vector, pTV00, and infiltrated *N. benthamiana* plants with *Agrobacterium* containing each plasmid. TRV:NbRabE1(F), TRV:NbRabE1(N), and TRV:NbRabE1(C) contain the 648-bp full-length coding region, 310-bp N-terminal region, and 307-bp C-terminal region of the cDNA, respectively (Fig. 3a). VIGS with all of these TRV:NbRabE1 constructs resulted in growth arrest, abnormal leaf development, and premature senescence (Fig. 3b). The effect of gene silencing on the level of *NbRabE1* mRNA was examined by real-time quantitative RT-PCR using primers corresponding to the 3′-UTR of the *NbRabE1* cDNA (Fig. 3c). Real-time

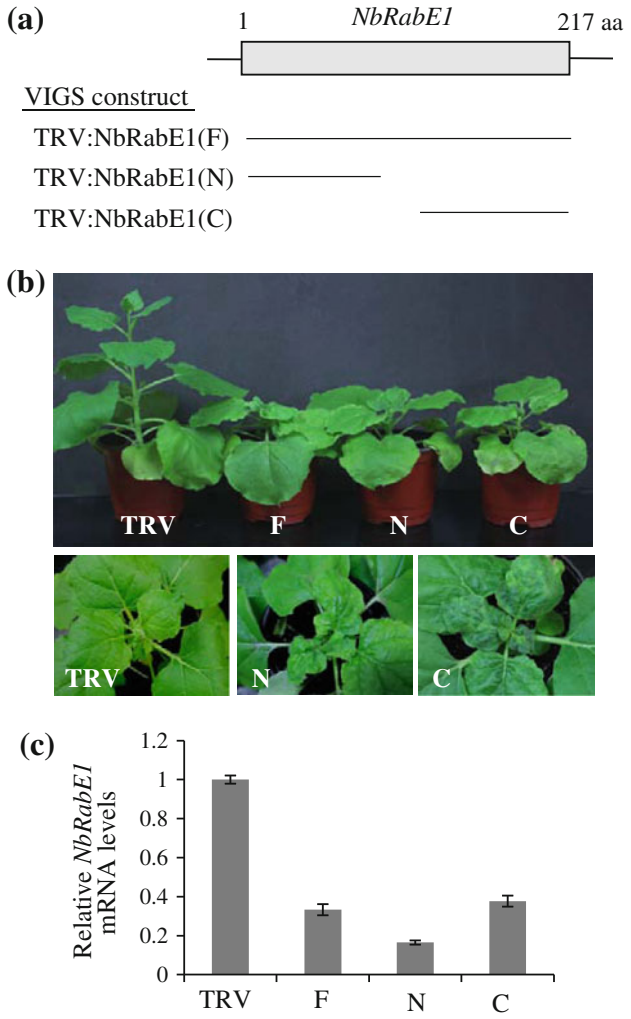

Fig. 3 VIGS constructs, phenotypes, and suppression of *NbRabE1* transcripts. **a** Schematic drawing of *NbRabE1* structure showing three VIGS constructs (*F*, *N*, and *C*) containing different *NbRabE1* cDNA fragments (indicated by *bars*). **b** Growth arrest, abnormal leaf development, and premature senescence phenotypes in TRV:NbRabE1 VIGS plants compared with TRV control plants at 20 days after infiltration (DAI). **c** Real-time quantitative RT-PCR analyses of *NbRabE1* transcript levels in the VIGS lines using *NbRabE1-UTR* primers that recognize the 3′-untranslated region of the *NbRabE1* cDNA. The mRNA levels of *β*-tubulin were used as a control

qRT-PCR detected significantly reduced levels of endogenous *NbRabE1* mRNA in all *NbRabE1* VIGS lines compared with TRV control, indicating silencing of *NbRabE1* in the VIGS lines (Fig. 3c).

Defects in stomata guard cell division in the *NbRabE1* VIGS plants

During stomatal development, symmetric division of a guard mother cell forms a ventral wall, which is then extensively remodeled to separate the two guard cells at the middle lamella and to form a stomatal pore (Bergmann and Sack 2007). A large number of stomata exhibited abnormal morphology in the TRV:NbRabE1(C) leaves (Fig. 4a, b). Defects in guard cell cytokinesis were the most frequently observed phenotypes: the abnormal stomata completely lacked pores or ventral walls (type 2), or possessed partial/incomplete walls without pores (type 3). Some stomata had a pore that was attached to one wall protrusion (type 4). Overall, more than 60 % of the examined stomata ($n = 200$) exhibited defective guard cell cytokinesis to varying degrees in the TRV:NbRabE1(C) leaves (Fig. 4d). In addition, incomplete cell walls were infrequently observed in epidermal cells of the TRV:NbRabE1(C) leaves (marked with asterisk in Fig. 4a). In contrast, these abnormalities were completely absent in TRV control leaves. Transmission electron microscopy revealed thickened cell walls of the pore-less stomata compared with normal stomata (Fig. 4c). Staining of the leaf epidermal layers with the neutral lipid-specific Nile blue A preferentially stained the thickest walls surrounding the pore in normal TRV stomata, suggesting preferential deposition of neutral lipids in the specific cell walls of the guard cell (Fig. 4e). In contrast, Nile blue A stained the entire cell wall of the abnormal pore-less stomata in *NbRabE1* VIGS lines, indicating abnormal uniform deposition of neutral lipids (Fig. 4e).

Nuclear morphology in the defective stomata

Nuclear numbers and morphology of stomata were investigated by DAPI staining of TRV and TRV:NbRabE1(C) leaves (Fig. 5a). All normal stomata (type 1) found in both TRV and TRV:NbRabE1(C) leaves contained one nucleus per guard cell. Most of the type 2 stomata in TRV:NbRabE1(C) leaves, which completely lacked pores or ventral walls, possessed two nuclei, indicative of defective guard cell cytokinesis; however, 11.7 % of the type 2 stomata contained only one nucleus (Fig. 5b). Type 3 stomata possessing partial/incomplete walls without pores were all binucleate. Thus, the binucleate phenotype seems to be positively correlated with the degree of ventral wall formation.

Premature senescence of the *NbRabE1* VIGS plants

To examine the effects of *NbRabE1* silencing at the ultrastructural level, we examined leaf cells from TRV control and TRV:NbRabE1(C) lines at 14 DAI using light microscopy and transmission electron microscopy. Transverse leaf sections revealed that both TRV control and TRV:NbRabE1(C) leaves had the typical leaf structure of dicotyledonous plants with dorsoventral organization of palisade mesophyll and spongy mesophyll cells (Fig. 6a, b).

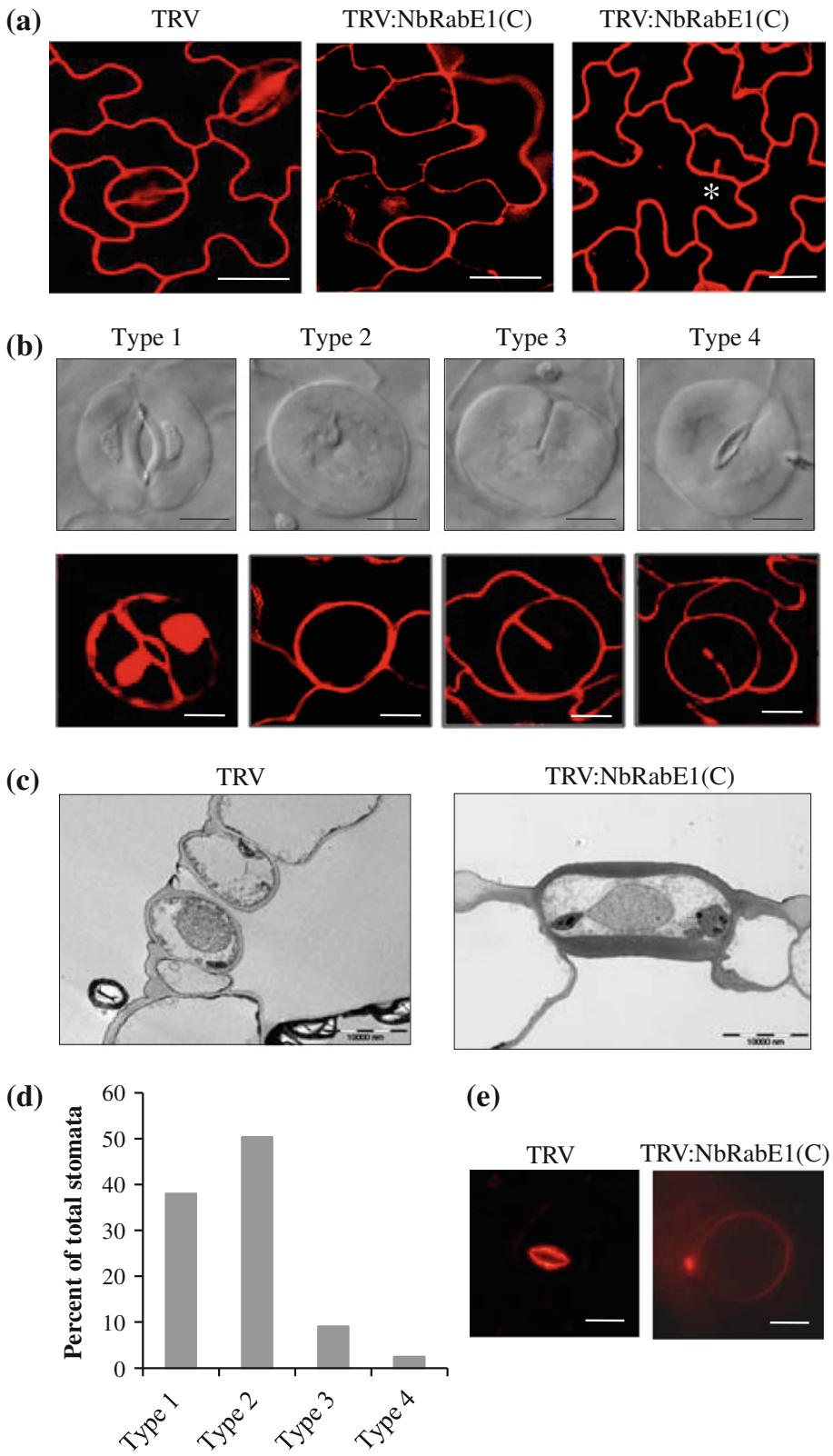

Fig. 4 Abnormal stomata development in TRV:NbRabE1 leaves. **a** Confocal laser scanning microscopy of propidium iodide (PI)-stained leaf abaxial epidermis from TRV and TRV:NbRabE1(C) lines. Pore-less stomata and incomplete cell walls (marked with *asterisk*) were observed in TRV:NbRabE1(C) leaves. *Scale bars* 20 μm. **b** Differential interference contrast optics (*top*) and PI-staining (*bottom*) of four different stomatal types in the abaxial epidermis of TRV:NbRabE1 leaves. *Scale bars* 10 μm. **c** Transmission electron microscopy (TEM) of a normal stoma from TRV control and a pore-less stoma from TRV:NbRabE1(C) leaves. The pore-less stoma exhibited a thickened cell wall. **d** The frequency of occurrence of the four different types of stomata ($n = 200$) in the abaxial epidermis of TRV:NbRabE1(C) leaves. **e** Nile blue A staining of a normal stoma from TRV control and a pore-less stoma from TRV:NbRabE1(C) leaves. Nile blue A stains neutral lipids to produce orange fluorescence. *Scale bars* 10 μm

However, the palisade and spongy mesophyll cells of TRV:NbRabE1(C) lines contained many dense granule-like structures (Fig. 6b, cf. control in Fig. 6a). Transmission electron microscopy revealed degeneration of chloroplasts and cellular compartments in TRV:NbRabE1(C) lines (Fig. 6e–j, cf. control in Fig. 6c, d). The cells accumulated a

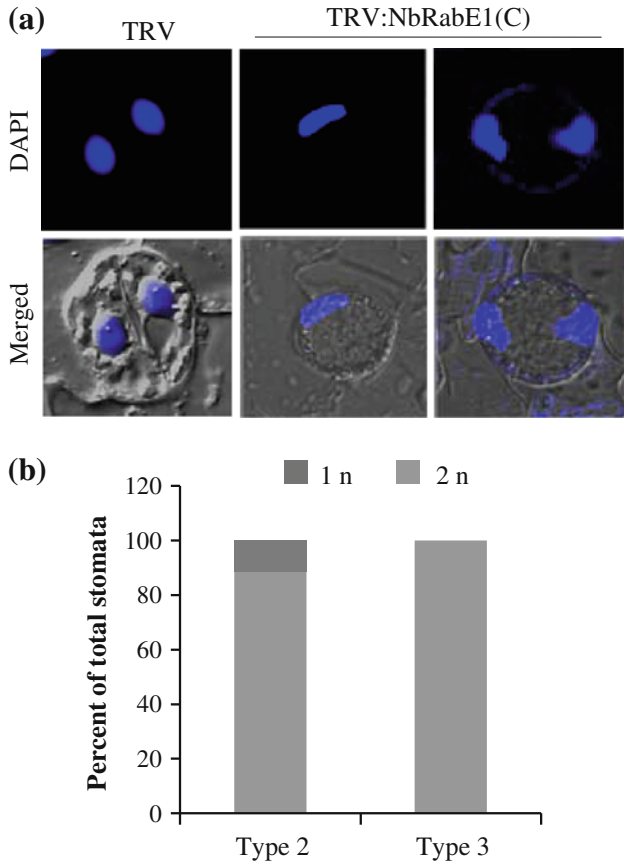

Fig. 5 Stomatal nuclear morphology. **a** Fluorescence microscopy of nuclear morphology in normal stomata from TRV control epidermis and in type 2 defective stomata from TRV:NbRabE1(C) epidermis after DAPI staining. The type 2 stomata contained either one or two nuclei. **b** The frequency of mononucleate and binucleate cells in type 2 and type 3 stomata in the abaxial epidermis of TRV:NbRabE1(C) leaves. Sixty stomata were counted for each type

large number of multivesiculated membrane structures and autophagosome-like vesicles containing parts of disintegrating chloroplasts and other compartments, suggesting that NbRabE1 depletion led to premature senescence of the cells (Fig. 6e–j, cf. control in Fig. 6c, d). Interestingly, a similar phenotype of accumulation of dense vesicles and autophagosome-like structures was reported for the embryonic lethal *vps9a-1* and *vacuoleless1* mutants, which are defective in Rab5 GTPase activation and vacuole biogenesis, respectively (Rojo et al. 2001; Goh et al. 2007).

Growth retardation and reduced root hair formation in NbRabE1(DN)-expressing *Arabidopsis* plants

To further investigate in vivo functions of NbRabE1, we generated transgenic *Arabidopsis* plants that express hemagglutinin (HA) tag-fused NbRabE1, NbRabE1(CA), and NbRabE1(DN) (Fig. 7). Immunoblotting with anti-HA antibodies detected the N-terminal HA in the fusion proteins and demonstrated the expression of the proteins in the transgenic lines (Fig. 7a). NbRabE1(DN)-expressing plants showed retardation of shoot and root growth compared with wild-type Col-0 plants, while NbRabE1- and NbRabE1(CA)-expressing plants were indistinguishable from the wild type (Fig. 7b–d). Furthermore, ectopic expression of NbRabE1(DN) inhibited root hair development by decreasing both the number and the length of root hairs (Fig. 8a–c). Root hair morphogenesis involves the rapid deposition of new wall materials and membranes via polarized secretion in response to developmental and environmental cues (Schiefelbein 2000). These results further suggest a critical function of RabE GTPases in plant growth and development. However, we did not observe any evidence of defective cytokinesis in root cells of the NbRabE1(DN)-expressing plants, such as binucleate cells, irregular cell files, or cell wall stubs. The stomatal defects shown in the *NbRabE1* VIGS *N. benthamiana* plants were also not observed in any of the transgenic plants.

Discussion

In plants, it has been predicted that RabE GTPases regulate secretory vesicle trafficking from the Golgi apparatus to the plasma membrane, thus, delivering newly synthesized proteins and materials to the cell surface and the cell wall (Nielsen et al. 2008; Woollard and Moore 2008). However, it is largely unknown whether or how RabE isoforms regulate polarized membrane trafficking during cytokinesis, cell plate formation, or cell expansion (Carter et al. 2004; Woollard and Moore 2008). In this study, we investigated protein characteristics and physiological functions of a new RabE isoform, NbRabE1, using VIGS in *N. benthamiana* and ectopic expression of NbRabE1 mutant forms in *Arabidopsis*. We observed previously uncharacterized phenotypes of RabE deficiency in plants, such as defective guard cell cytokinesis, reduced root hair formation, and premature senescence. The phenotype of growth retardation and abnormal leaf development also observed in the *NbRabE1* VIGS lines are consistent with the phenotype of the cosuppression lines of *Arabidopsis RabE1* genes (Speth et al. 2009). In *NbRabE1* VIGS leaves, stomata were irregularly shaped, lacking the ventral wall or displaying cell wall stubs, and most of the abnormal guard cells were binucleate. Incomplete cell walls were also observed in the leaf epidermal cells of the *NbRabE1*-silenced plants, albeit infrequently. Both guard cell cytokinesis and root hair growth involve the deposition of new cell wall materials and new membrane to a specific site through polarized secretion (Ryan et al. 2001; Söllner et al. 2002). NbRabE1 dysfunction may interfere with execution of guard cell cytokinesis and root hair development by

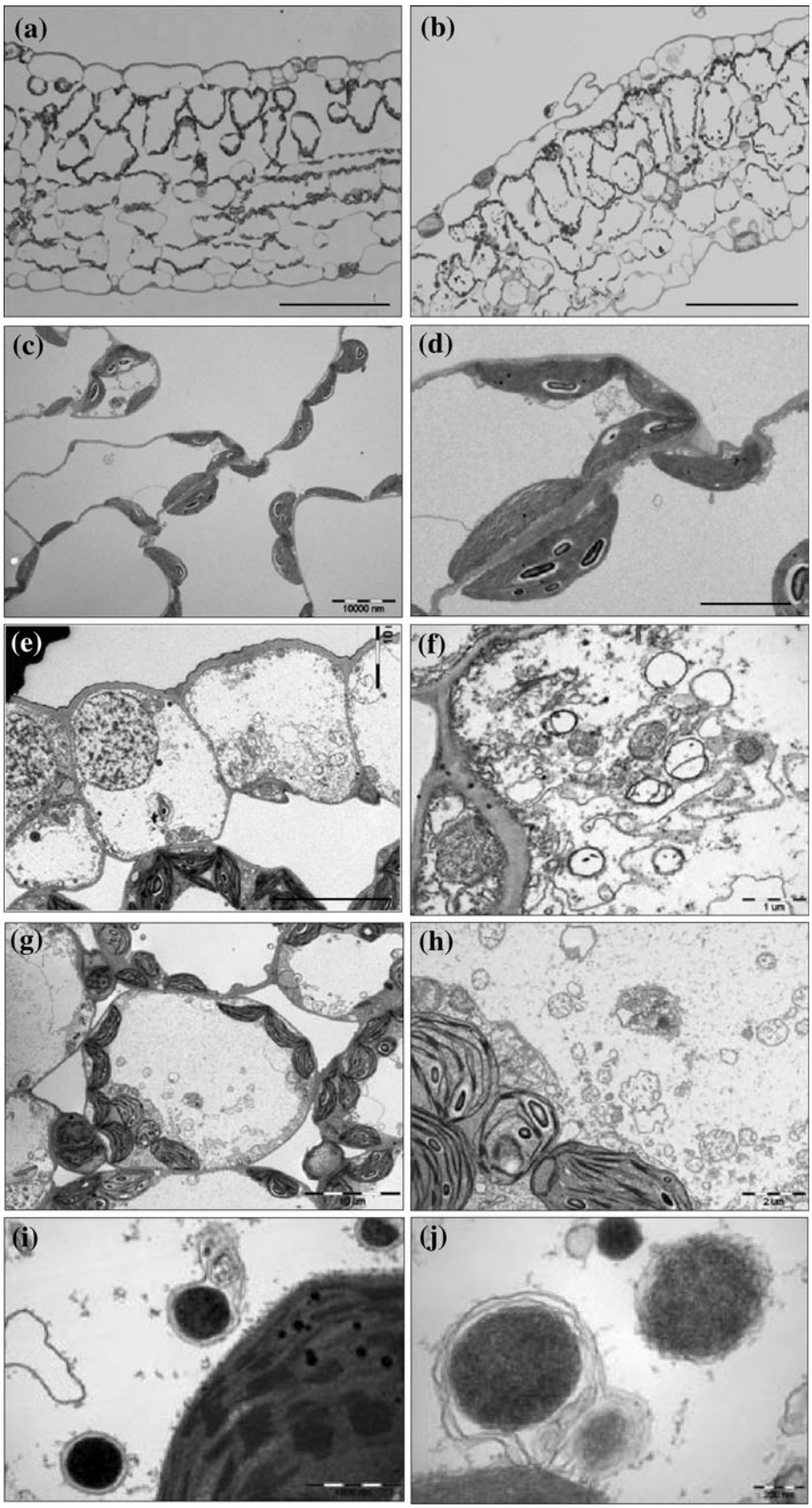

Fig. 6 Cellular phenotypes of premature senescence. Leaf sections from TRV (**a**) and TRV:NbRabE1(C) (**b**) lines were examined by light microscopy. *Scale bars* 100 μm. **c–j** Representative TEM images. A spongy mesophyll cell (**c**) and its chloroplasts (**d**) of TRV control at 14 DAI; degenerating cellular compartments in epidermal cells (**e**, **f**) and in spongy mesophyll cells (**g**, **h**) of TRV:NbRabE1(C) leaves at 14 DAI; autophagosome-like vesicles containing parts of degrading chloroplasts (**i**, **j**) of TRV:NbRabE1(C) leaves at 14 DAI. Images **d**, **f** and **h** are enlarged photos of part of **c**, **e** and **g**, respectively. *Scale bars* 100 μm (**a**, **b**); 10 μm (**c**, **e**, **g**); 5 μm (**d**); 2 μm (**h**); 1 μm (**f**); 0.5 μm (**i**); and 0.2 μm (**j**)

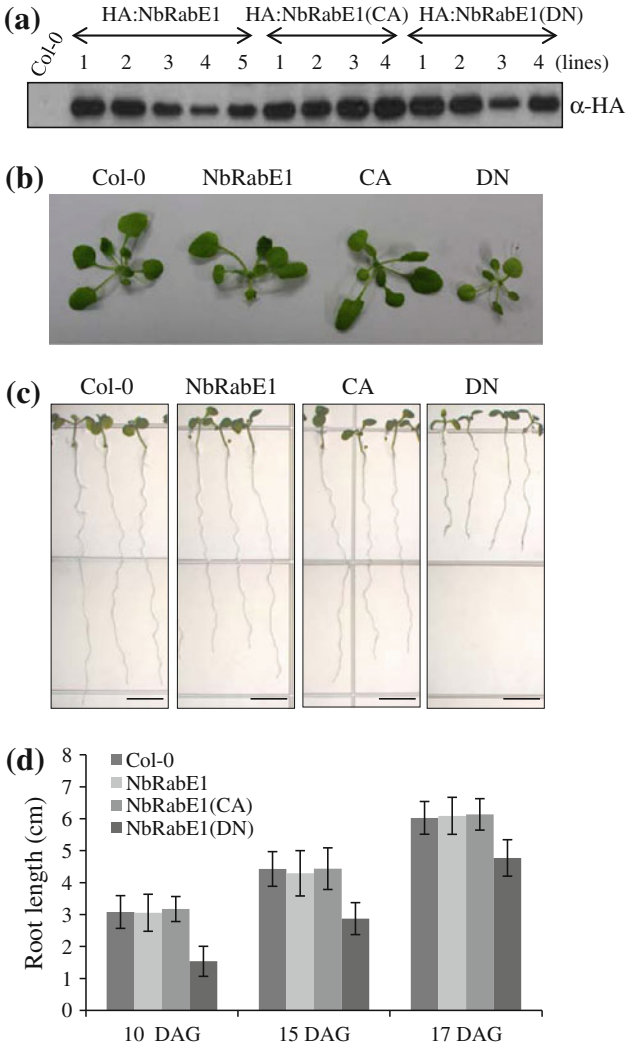

Fig. 7 Phenotypes caused by ectopic expression of wild-type and mutant NbRabE1 in *Arabidopsis*. **a** Immunoblotting with anti-HA antibodies to measure expression of HA:NbRabE1, HA:NbRabE1(CA), and HA:NbRabE1(DN) fusion proteins in multiple *Arabidopsis* transgenic lines. **b** Retarded plant growth of NbRabE1(DN)-expressing plants. The plants were soil-grown and the aerial part was photographed at 30 days after sowing. Representative photos are shown. **c** Retarded root growth of NbRabE1(DN)-expressing plants, which were grown vertically on the surface of MS medium, at 10 days after germination (DAG). *Scale bars* 5 mm. **d** Root length was measured in seedlings shown in **c** at 10, 15, and 17 DAG. Each data point represents the mean ± standard deviation (SD) ($n = 20$ seedlings)

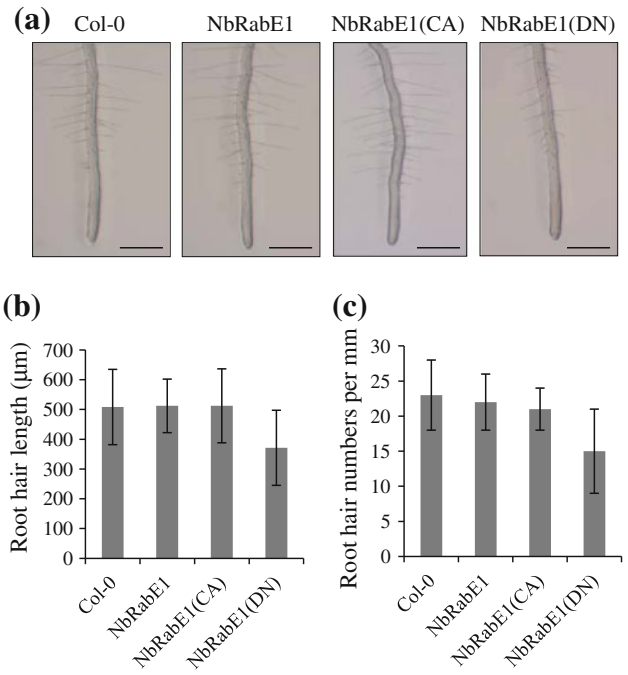

Fig. 8 Reduced root hair formation in NbRabE1(DN)-expressing *Arabidopsis* plants. **a** Reduced root hair development in NbRabE1(DN)-expressing plants at 10 DAG. Representative photos are shown. *Scale bars* 1 mm. **b** Root hair length was measured in ten seedlings at 10 DAG. Each data point represents the mean ± SD ($n = 200$ root hairs). **c** Root hair numbers per mm were measured in ten seedlings at 10 DAG. Visible root hairs were counted in a 1-mm section in the root elongation zone. Each data point represents the mean ± SD ($n = 200$ root hairs)

hampering polarized membrane trafficking and localized release of cell wall materials and membranes, causing defects in cell plate assembly and polarized cell expansion. In previously characterized cytokinesis-defective mutants, the most notable cytokinesis defects were observed in stomata, suggesting that stomatal development is particularly sensitive to disruptions during cytokinesis (Yang et al. 1999; Söllner et al. 2002; Falbel et al. 2003; Haga et al. 2007). In addition to displaying stomatal defects, some of these mutants also exhibited defective root hair morphogenesis (Söllner et al. 2002; Falbel et al. 2003).

Divisions of guard mother cells produce guard cells in mirror-like symmetry during leaf development (Nadeau and Sack 2002). A gradient of guard cell cytokinesis defects has been detected in *Arabidopsis* mutants such as *cyd1* (Yang et al. 1999), *scd1-1* (Falbel et al. 2003), *exo84b* (Fendrych et al. 2010), *myb3r1–myb3r4* double mutants (Haga et al. 2007), and other cytokinesis-defective mutants (Söllner et al. 2002). The guard cell cytokinesis defects have also been observed in transgenic tobacco plants overexpressing the kinase-negative mutant form of NPK1, the cell plate-associated mitogen-activated protein kinase kinase kinase (MAP3K) (Nishihama et al. 2001), and in *N. benthamiana* plants after VIGS of *NPK1* (Jin et al. 2002). Many of these mutants also exhibit abnormal nuclear morphology in the aberrant guard cells, indicating abnormal/incomplete separation of daughter nuclei after division (Yang et al. 1999; Nishihama et al. 2001; Jin et al. 2002). Among these, stomatal cytokinesis-defective 1 (SCD1) is particularly interesting, because its function may be related to the regulation of Rab family proteins (Falbel et al. 2003). The *scd1* mutants exhibited defects in guard

cell cytokinesis, root elongation, and root hair development similar to the phenotype of NbRabE1 dysfunction. It has been hypothesized that SCD1 regulates the function of Rab GTPases required for trafficking of specific secretory vesicles during cell plate formation and polarized cell expansion (Falbel et al. 2003). SCD1 has two domains, an N-terminal DENN domain and C-terminal WD-40 repeats. Various DENN domain proteins in metazoa directly interact with Rab GTPases and function as Rab-specific guanine-nucleotide exchange factor (GEF) to activate specific Rab isoforms (Marat et al. 2011). Furthermore, it has been proposed that DENN domains may be at the interface between different Rab pathways by interacting with one Rab while functioning as a GEF for another Rab (Marat et al. 2011). It would be interesting to investigate whether SCD1 is linked to RabE family GTPases in regulating membrane trafficking of cell wall polysaccharides and proteins to the cell surface and to the division plane during guard cell cytokinesis.

Although *NbRabE1* was targeted for VIGS, expression of its homologs in *N. benthamiana* was expected to be affected as well. Previously, cosuppression using the *RabE1d* sequence in *Arabidopsis* resulted in differential silencing of the five RabE1 family genes without affecting expression of the RabD family genes: *RabE1d* and *RabE1e* were significantly silenced followed by silencing of *RabE1b* and *RabE1c*, while *RabE1a* was only slightly silenced (Speth et al. 2009). Since *NbRabE1* shared significant sequence homology with *Arabidopsis RabE1a*, *RabE1b* and *RabE1c* (Fig. 1a), VIGS with the *NbRabE1* sequence is expected to preferentially downregulate the expression of *N. benthamiana* homologs of this branch of *RabE1* genes, and thus the stomatal defects and abnormal growth observed in the *NbRabE1* VIGS lines are likely caused by silencing of multiple *NbRabE* genes. Ectopic expression of the dominant-negative mutant of NbRabE1 in *Arabidopsis* resulted in growth retardation and defective root hair formation, but no defects in root cell division or stomatal development. We speculate that the presence of functional *Arabidopsis* RabE proteins in the transgenic plants possibly mitigated cellular effects of NbRabE1(DN) expression. Nonetheless, taken together, these results suggest that RabE GTPases play an important role in diverse developmental processes of plants.

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