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REVIEW

Role of an *Arabidopsis* Rab GTPase RabG3b in Pathogen Response and Leaf Senescence

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Abstract In our previous proteomic analysis, we isolated a small GTPase RabG3b as a salicylic acid-responsive protein in *Arabidopsis* (Oh et al. in Plant Cell 17:2832–2847, 2005). Here, we constructed transgenic plants over-expressing wild-type (RabG3bOX), constitutively active (RabG3bCA), and dominant negative (RabG3bDN) forms of *RabG3b* for functional studies. The phenotypes of these transgenic plants were indistinguishable from wild-type plants under normal growth conditions. However, both RabG3bOX and RabG3bCA plants displayed unrestricted hypersensitive programmed cell death against a fungal toxin Fumonisin B1 and a fungal pathogen *Alternaria brassicicola*, whereas no major difference between wild-type and RabG3bDN plants was observed. In addition, RabG3bOX and RabG3bCA plants underwent accelerated leaf senescence compared to wild-type and RabG3bDN plants. These results suggest that RabG3b is a modulator for cell death progression during pathogen response and senescence process in plants.

Keywords Rab GTPase · Programmed cell death · Hypersensitive response · Leaf senescence · Fumonisin B1 · *Alternaria brassicicola*

Introduction

Plants have developed highly complex signaling and defense mechanisms to protect themselves against pathogen attacks. Plant disease resistance is often manifested by programmed cell death (PCD) termed the hypersensitive response (HR) that depends on interactions between products of plant resistance (*R*) and pathogen avirulence (*avr*) genes, which is referred to as a gene-for-gene interaction (Grant et al. 2006; Hofius et al. 2007; Kim et al. 2008). HR-PCD occurs rapidly (<12 h) upon attempted pathogen infection and prevents the spread of pathogens, thereby regulating both immunity and disease progression in plants (Goodman and Novacky 1994; Lincoln et al. 2002; Abramovitch and Martin 2004; del Pozo et al. 2004; Greenberg and Yao 2004; Grant et al. 2006). A series of cell death-inducing events, including fortification of the cell wall, accumulation of reactive oxygen species (ROS), expression of pathogenesis-related (*PR*) genes, and production of phytoalexin, occur upon pathogen attack (Greenberg and Ausubel 1993; Dietrich et al. 1994; Rate et al. 1999). Pathogen-derived phytotoxic compounds act as external virulence factors that elicit HR-PCD. Necrotrophic phytopathogenic fungi such as *Alternaria* and *Fusarium* spp. can produce mycotoxins such as Fumonisin B1 (FB1; Desjardins et al. 1995; Gilchrist et al. 1995; Dutton 1996; Gilchrist 1997). FB1, a sphinganine analog that inhibits sphingolipid biosynthesis, elicits an apoptotic form of PCD in both plant and animal cells (Wang et al. 1990, 1996; Abbas et al. 1994; Gilchrist et al. 1995; Tolleson et al. 1996; Yoo et al. 1996; Liang et al. 2003) and has been used in an *Arabidopsis* protoplast system to investigate cell death signaling events (Asai et al. 2000).

Plant PCD also takes place during various processes of plant growth and development, including leaf senescence, degeneration of aleurone layer, and development of endosperm and tracheary element (Young and Gallie 2000; Dominguez et al. 2004; Fukuda 2004; van Doorn and Woltering 2004; Lim et al. 2007; Turner et al. 2007).

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Leaf senescence is the final stage of leaf development and characterized by PCD associated with degradation of intracellular organelles and macromolecules for nutrient recycling (Buchanan-Wollaston et al. 2003; van Doorn and Woltering 2004; Lim et al. 2007). The complicated process of senescence is under genetic control, as demonstrated by expression of a large number of senescence-associated genes (*SAGs*) during senescence (Yoshida 2003). Many *SAGs* are differentially expressed in developing tissues and induced by internal or external signaling factors such as plant hormones, ozone, and UV radiation (Park et al. 1998; Weaver et al. 1998). Molecular and genetic studies have revealed regulatory factors of senescence that include NAC and WRKY transcription factors (Guo and Gan 2006; Lim et al. 2007).

To identify novel proteins involved in pathogen defense, we previously performed a comprehensive analysis of the *Arabidopsis* secretome (Oh et al. 2005). From this analysis, a small GTP-binding protein RabG3b was identified as one of the SA-responsive proteins. Compared to wild-type and transgenic plants overexpressing dominant negative *RabG3b* (RabG3bDN), transgenic plants overexpressing wild-type (RabG3bOX) and constitutively active *RabG3b* (RabG3bCA) showed expanded HR-PCD upon infection and accelerated leaf senescence. These results suggest that RabG3b may regulate PCD associated with pathogen response and senescence.

Materials and Methods

Plant Materials

Arabidopsis thaliana plants (Col-0) were grown in a growth room at 24°C under long-day conditions (16-h light/8-h dark cycle) or in a growth chamber under short-day conditions (8-h light/16-h dark cycle) for pathogen-induced disease assays. To generate transgenic plants overexpressing *RabG3b*, PCR products amplified using *RabG3b* (At1g22740) cDNA were cloned into the binary vector pBI121 under the control of the CaMV 35S promoter. Point mutations of *RabG3b* to generate RabG3bCA (RabG3b[Q67L]) and RabG3bDN (RabG3b[T22N]) were created by PCR using the following primers: for *RabG3bCA*, 5′-CAA ATA TGG GAC ACT GCT GGG CTA GAG AGG TTT CAA AGT CTT G-3′ and 5′-CAA GAC TTT GAA ACC TCT CTA GCC CAG CAG TGT CCC ATA TTT G-3′; for *RabG3bDN*, 5′-GAG ACA GCG GGG TTG GCA AAA ACT CGT TGA TGA ATC AAT ATG TG-3′ and 5′-CAC ATA TTG ATT CAT CAA CGA GTT TTT GCC AAC CCC GCT GTC TC-3′. *Arabidopsis* plants were transformed using the flower dip method (Clough and Bent 1998).

Plant Treatments

For leaf senescence assays, leaves of 4-week-old plants were detached and floated on deionized water at room temperature in the dark and photographed at 1, 3, and 7 days (Liu et al. 2005). *Alternaria brassicicola* was inoculated on leaves of 4-week-old plants by applying a 10-μl drop of spore suspension (5×10^5 spores/ml; Oh et al. 2005). Inoculated plants were kept at 100% relative humidity in a growth chamber for the indicated times. To count the number of spores, ten leaves were collected and vigorously shaken in 5 ml of 0.1% Tween 20 in a test tube. Leaves were removed, and the spore-containing suspension was centrifuged at 5,000×*g* for 15 min. The spores were resuspended in 200 μl of 0.1% Tween 20, serially diluted, and counted with a microscope. The lesions and fungal hyphae were visualized by staining the infected leaves according to a previously described method (Oh et al. 2005). Detached leaves treated with *A. brassicicola* for 4 days were stained with lactophenol-aniline blue. For FB1 (Sigma-Aldrich) treatment (Watanabe and Lam 2006), leaves of 4-week-old plants grown in soil were infiltrated with 10 μM FB1 using 1 ml syringe without needle and were kept in a growth chamber for up to 3 days.

H_2O_2 Staining

For in vivo H_2O_2 measurements, protoplasts isolated from leaves were incubated in 2 μM H_2DCFDA (Molecular Probes) for 2 min. Protoplasts were transferred to microscope slides and observed using a confocal microscope (Zeiss LSM 510 META) at 488–505 nm to visualize green fluorescence of the H_2O_2-oxidized probe. H_2O_2 was detected by an endogenous peroxidase-dependent in situ histochemical staining procedure using 3,3′-diaminobenzidine (DAB). Leaves were placed in a solution containing 1 mg/ml DAB (pH 5.5) for 2 h at room temperature, boiled in 95% ethanol for 2 min, and stored in distilled water. H_2O_2 production was visualized as a reddish-brown coloration. Images were captured using a microscope with a charge-coupled device camera (Leica EZ4D).

Trypan Blue Staining

Trypan blue staining was performed as previously described (Bowling et al. 1997). Samples were submerged in lactic acid–phenol–trypan blue solution (2.5 mg/ml trypan blue, 25% lactic acid, 23% water-saturated phenol, and 25% glycerol) and boiled for 1 min. Stained samples were then placed in a 60% chloral hydrate solution and finally equilibrated with 50% glycerol.

RNA Analysis

RT-PCR was performed with 1 μg total RNA (or 100 ng polyA$^+$ RNA) extracted from various *Arabidopsis* tissues using the Reverse Transcription System (iNtRON). Transcripts were amplified by PCR using the following primers: for *RabG3b*, 5′-AGA AGG CTA GAG AAT GGT GTG CTG A-3′ and 5′-CAT ACA AAT CGT GAA CCA CCA AAT G-3′; for *AtSEN1*, 5′-ACA TCA CGA ATT GGA AAC TGG TCA-3′ and 5′-TTG ACC GCT CTC ACA ACC GAT TAT-3′; for *UBQ10*, 5′-GAT CTT TGC CGG AAA ACA ATT GGA GGA TGG T-3′ and 5′-CGA CTT GTC ATT AGA AAG AAA GAG ATA ACA GG-3′.

Protein Analysis

The full-length coding region of *RabG3b* was cloned into the pET15 vector (Novagen) and used for transformation of *Escherichia coli* (BL21) cells. Production of recombinant RabG3b proteins was induced by the addition of 1 mM IPTG for 2 h, and the proteins were purified using an Ni$^+$–NTA column according to the manufacturer's instructions (Qiagen). Affinity-purified recombinant proteins were used to raise an anti-RabG3b antibody in rabbits. Western blot analysis was performed using standard protocols (Lee et al. 2004). Proteins were separated on 12% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and probed with the anti-RabG3b antibody overnight at 4°C. Antibody-bound proteins were detected after incubation with secondary antibody conjugated to horseradish peroxidase using the ECL system (Amersham Biosciences).

Scanning Electron Microscopy

Samples were fixed in FAA solution (10% formaldehyde, 5% acetic acid, and 45% ethanol) overnight under vacuum, dehydrated through a graded ethanol series, and dried using a Critical Point Dryer (Tousimis Research Corp). Samples were then mounted on stubs, coated with gold on a sputter-coater (Cressington Scientific Instruments), and observed using a scanning electron microscope (JEOL JSM 5300).

Results and Discussion

Identification of RabG3b as an *Arabidopsis* Salicylic Acid-Responsive Secreted Protein

In our previous work, we analyzed SA-responsive changes in the *Arabidopsis* secretome to identify secreted proteins involved in plant–pathogen interactions (Oh et al. 2005). Of the identified proteins, RabG3b (At1g22740) was selected for further characterization. RabG3b shows strong sequence similarity to the Ypt/Rab family members of yeasts and mammals and contains conserved motifs for GTP-binding and hydrolysis (Fig. 1; Rutherford and Moore 2002).

Small GTPases are molecular switches that are activated by GTP binding and inactivated by the hydrolysis of GTP to GDP. In an active state, small GTPases interact with various downstream effector proteins and regulate signaling pathways (Yang 2002). The *Arabidopsis* genome contains 93 genes encoding small GTPases that regulate diverse cellular processes such as cell proliferation, cytoskeletal organization, and intracellular membrane trafficking. Phylogenetic analysis indicates that *Arabidopsis* contains Rop, Arf, Ran, and Rab, but not Ras, GTPases (Vernoud et al. 2003). RabG3b is a member of the RabG family, one of eight Rab subfamilies (RabA to RabH; Rutherford and Moore 2002; Vernoud et al. 2003). RabG GTPases are related to the animal Rab7 and yeast Ypt7, which generally function in the regulation of late steps in membrane trafficking from early endosome to late endosome and fusion with the lysosome or vacuole (Rutherford and Moore 2002; Vernoud et al. 2003). A recent study by Mazel et al. (2004) revealed that overexpression of *Arabidopsis RabG3e*

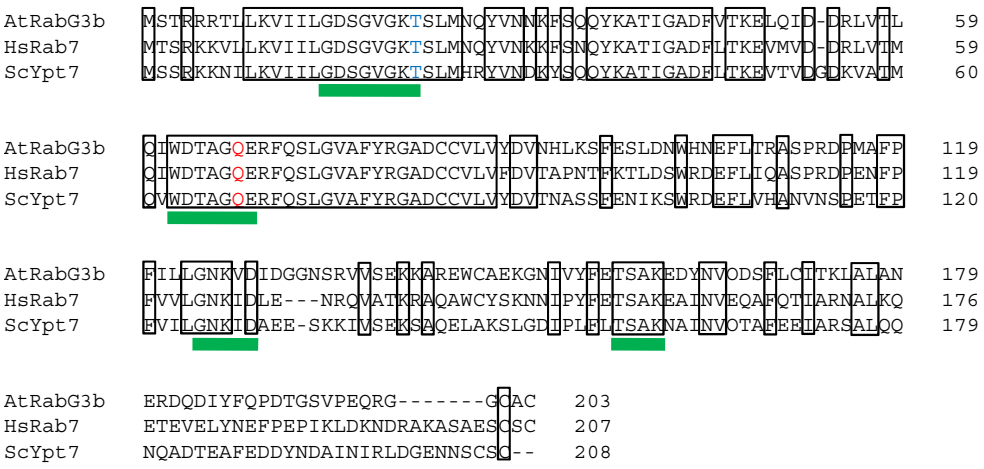

Fig. 1 Alignment of the amino acid sequence of *Arabidopsis* RabG3b (*AtRabG3b*) with human Rab7 (*HsRab7*) and yeast Ypt7 (*ScYpt7*). Sequences were aligned using ClustalW. Identical residues in all three organisms are *boxed*. *Green underlines* indicate sequence motifs involved in nucleotide binding and hydrolysis that are conserved in Rab GTPases. The *residues in blue and red* amino acid were mutated to generate the DN (T22N) and CA (Q67L) forms of RabG3b

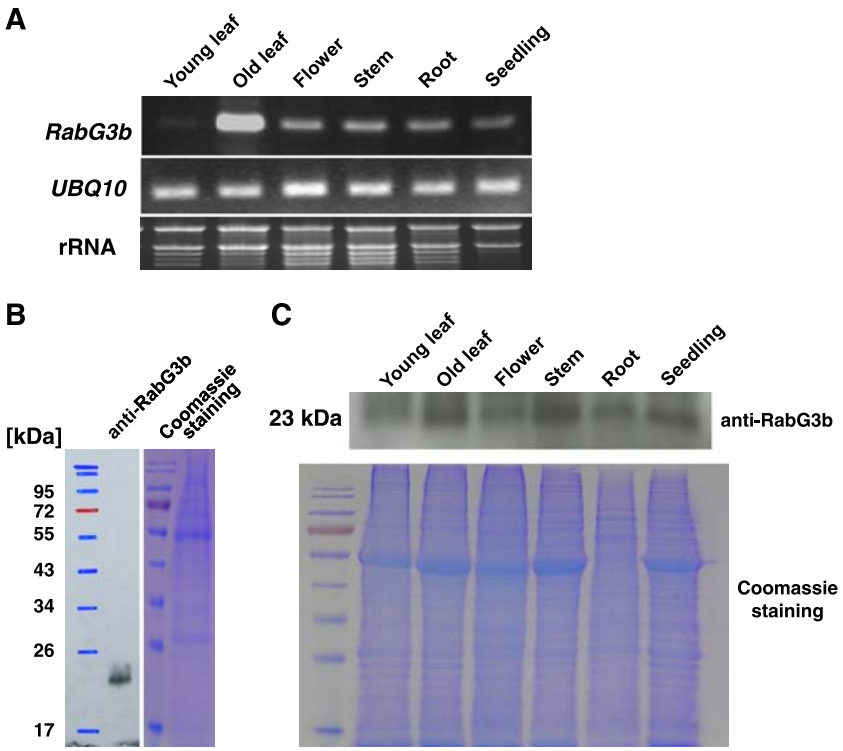

Fig. 2 Expression of RabG3b in *Arabidopsis* tissues. **a** Semi-quantitative RT-PCR analysis of *RabG3b* expression in tissues. *UBQ10* and rRNA served as controls. **b** Test for specific binding of the anti-RabG3b antibody to RabG3b proteins in plant extracts. For Western blot analysis, 25 μg of total proteins from 4-week-old plant leaves were used. **c** Western blot analysis of RabG3b expression in tissues. Each lane was loaded with 30 μg of total proteins from the indicated tissues

stimulates endocytosis and confers resistance to salinity and oxidative stress. Additionally, a tonoplast-localized rice Rab7 homologue is up-regulated in response to salt, cold, and drought stress, suggesting a role for Rab7-dependent regulation of endocytosis in plant adaptation to environmental stress (Nahm et al. 2003). In mammalian cells, Rab7 mediates the regulated internalization and degradation of nutrient transporters and triggers nutrient starvation that helps to induce cell death (Edinger et al. 2003).

The expression of RabG3b in tissues was determined by RT-PCR and Western blot analyses (Fig. 2). Both *RabG3b* transcripts and RabG3b proteins were detected in all tissues and, predominantly, in old leaves. Interestingly, microarray data available at Genevestigator (http://www.genevestigator.ethz.ch/; Zimmermann et al. 2004) indicate that *RabG3b* is highly induced during the PCD/senescence (Fig. 3),

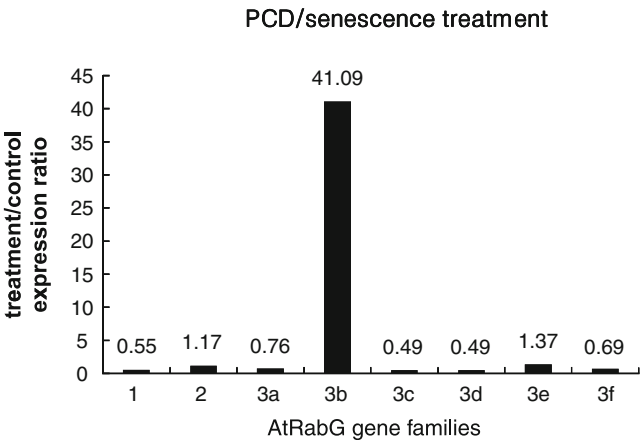

Fig. 3 Graphic analysis of transcript levels of *Arabidopsis RabG* genes during PCD and senescence. These microarray data are available at Genevestigator database (http://www.genevestigator.ethz.ch/)

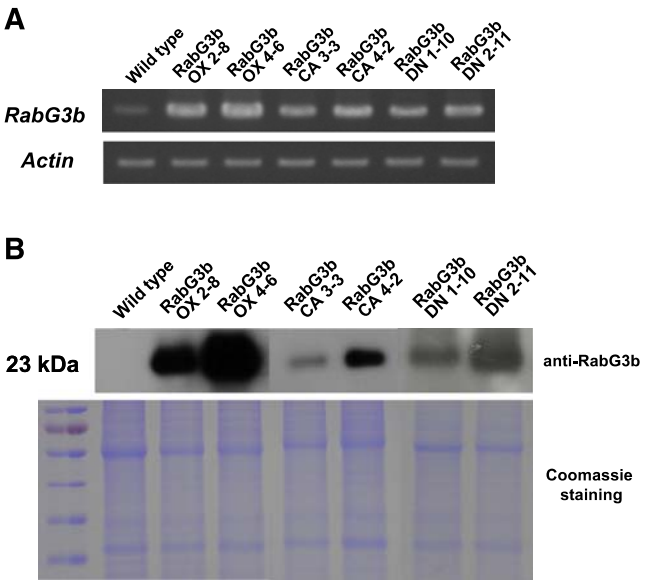

Fig. 4 Construction of RabG3bOX, RabG3bCA, and RabG3bDN transgenic *Arabidopsis* plants. Each two lines of RabG3bOX (2–8 and 4–6), RabG3bCA (3–3 and 4–2), and RabG3bDN (1–10 and 2–11) plants were selected for functional studies. **a** RT-PCR analysis of *RabG3b* expression in WT and RabG3b transgenic plants (RabG3-bOX, RabG3bCA, and RabG3bDN). *Actin* was used as a control. **b** Western blot analysis of RabG3b expression in WT and RabG3b transgenic plants. Each lane was loaded with 10 μg of total proteins

suggesting that RabG3b may be involved in PCD and senescence processes.

Generation of Transgenic Plants Overexpressing Wild-Type, Constitutively Active, and Dominant Negative *RabG3b*

To determine the function of RabG3b, we generated RabG3bOX, RabG3bCA, and RabG3bDN transgenic plants that overexpress either wild-type or mutant forms of *RabG3b*. The CA (Q67L) and DN (T22N) mutations result in GTP-bound and GDP-bound RabG3b, respectively (Fig. 4). We examined transcript expression level and protein induction using RT-PCR and Western blotting to confirm these transgenic plant lines of RabG3b (Fig. 4). For each construct, two homozygous lines (T3) exhibiting transgene overexpressions were chosen for functional analysis. RabG3bOX, RabG3bCA, and RabG3bDN transgenic plants were morphologically normal like wild-type plants.

Function of RabG3b in Fumonisin B1 and Pathogen Responses

Since microarray data from Genevestigator suggest that RabG3b may function in PCD and senescence, we investigated whether RabG3b transgenic plants display altered responses to the well-known PCD-eliciting mycotoxin FB1. RabG3bOX and RabG3bCA plant leaves infiltrated with FB1 displayed spreading cell death, in contrast to wild-type and RabG3bDN leaves which formed lesions restricted to the infiltration site (Fig. 5a). Cell death and H_2O_2 accumulation were strongly induced in the FB1-

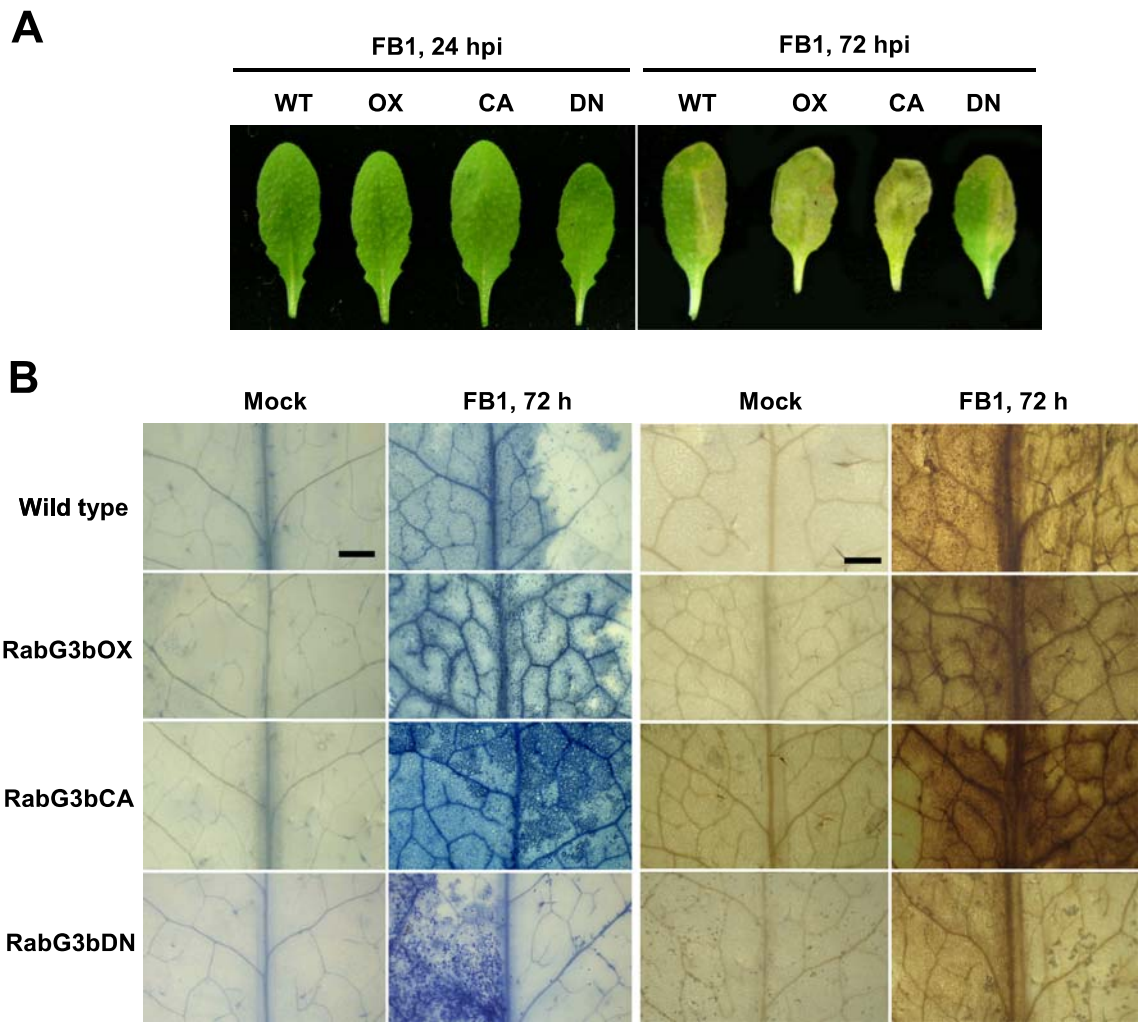

Fig. 5 RabG3b functions in cell death response upon FB1 treatment. **a** Phenotypes of leaves treated with FB1 for 24 h and 72 h. **b** Cell death in leaves stained with trypan blue (*left panels*) and H_2O_2 production in leaves stained with DAB (*right panels*). Staining was performed after treatments with mock (10 mM $MgSO_4$) and 10 μM FB1 for 3 days. *Bar* 1 mm

treated RabG3bOX and RabG3bCA leaves as determined by lactophenol blue and DAB staining (Fig. 5b).

We additionally examined the visible phenotypes of wild-type, RabG3bOX, RabG3bCA, and RabG3bDN plants inoculated with the fungal pathogen *A. brassicicola* (Fig. 6a). In contrast to wild-type and RabG3bDN plants, RabG3bOX and RabG3bCA plants displayed susceptible phenotypes that include the formation of spreading lesions, heavy colonization by fungal hyphae, and large production of fungal spores at the infection sites (Fig. 6b–d). It was noticeable that RabG3bOX and RabG3bCA plants developed expanded cell death at 24 h after infection with *A. brassicicola*. These results together suggest that accelerated and expanded lesion formation triggered by FB1 and *A. brassicicola* treatments in RabG3bOX and RabG3bCA plants is likely due to cell death associated with HR-PCD.

Function of RabG3b in Senescence

Leaf senescence was then examined in wild-type, RabG3bOX, RabG3bCA, and RabG3bDN plants (Fig. 7). Detached leaves from RabG3bOX and RabG3bCA plants

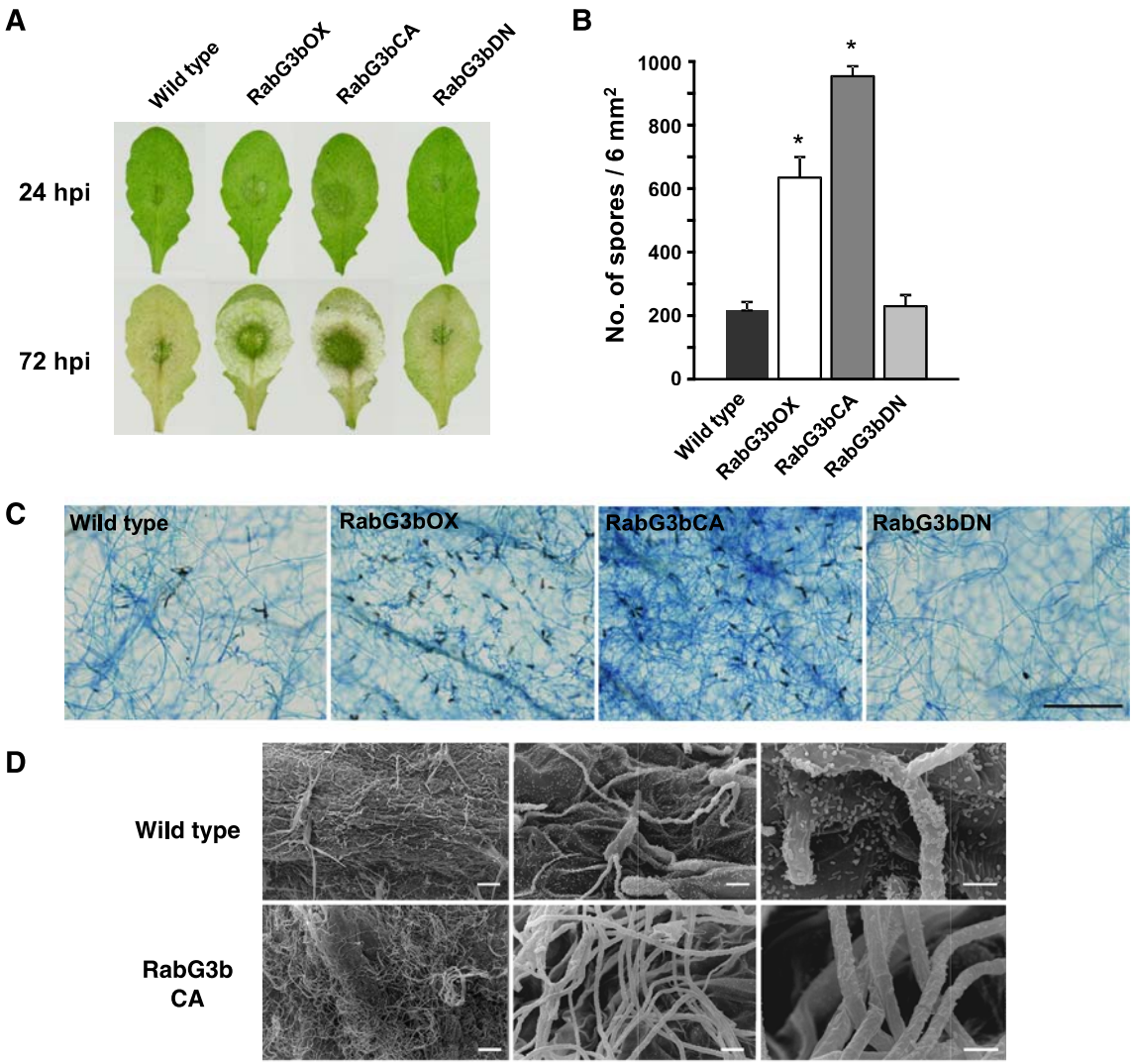

Fig. 6 RabG3b functions in cell death response upon *A. brassicicola* treatment. **a** Phenotypes of leaves inoculated with a 10-μl drop of *A. brassicicola* spore suspension (5×10^5 spores/ml). **b** Quantitation of newly formed spores in leaves inoculated as in **a**. Results represent means ($n=10$) of three independent experiments. *Asterisks* indicate a significant difference compared to WT ($P<0.01$). **c** Lesions stained with lactophenol blue. The formation of fungal mycelia was observed under a microscope. *Bar* 200 μm. **d** Growth of *A. brassicicola* observed by SEM. *Bars* 100 μm (*left*), 10 μm (*middle*), and 5 μm (*right*). Leaves were challenged with a 10-μl drop of spore suspension (5×10^5 spores/ml) of *A. brassicicola*

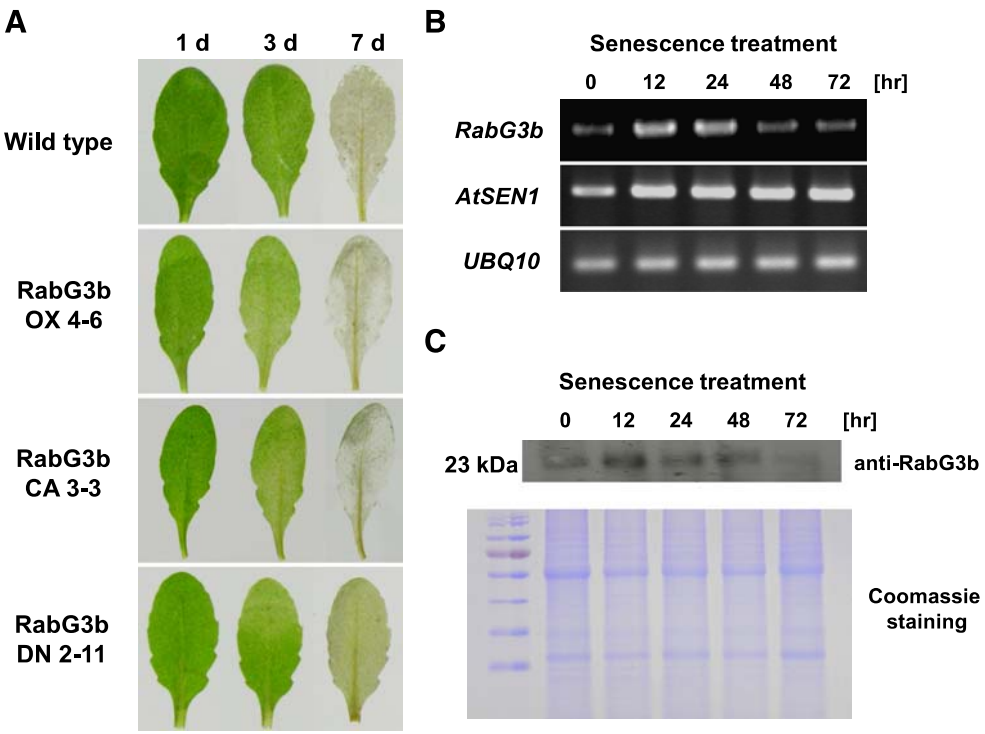

Fig. 7 RabG3b functions in leaf senescence. **a** Senescence assays. Leaves were detached, floated on water at room temperature in the dark, and photographed after 1, 3, and 7 days. Results were reproduced in five independent experiments using ten or more plants in each experiment. **b** Semiquantitative RT-PCR analysis of *RabG3b* and *AtSEN1*, a senescence marker, during senescence treatment. *UBQ10* was used as a control. Results were reproduced in three independent experiments. **c** Western blot analysis of RabG3b expression during senescence treatment

exhibited accelerated senescence compared to those of wild-type and RabG3bDN plants (Fig. 7a). Transcript levels of *RabG3b* in wild-type leaves were enhanced during the 12- to 24-h period of senescence (Fig. 7b). *AtSEN1*, a senescence marker gene, was also highly expressed during the course of senescence. A similar induction pattern of RabG3b protein was observed in the same time period (Fig. 7c). These results suggest that RabG3b plays a role in leaf senescence.

In this study, overexpression of wild-type and CA forms of *RabG3b* in plants resulted in accelerated leaf senescence and an expanded HR-PCD response to the FB1 fungal toxin and *A. brassicicola*. These phenotypes may be similar to those of the autophagy-defective mutants *ATG7* and *ATG9* in *Arabidopsis* and *ATG6*/*Beclin1* in tobacco and *Arabidopsis* (Doelling et al. 2002; Hanaoka et al. 2002; Liu et al. 2005; Patel and Dinesh-Kumar 2008). Plants deficient in *Beclin1* and other *ATG* genes underwent uncontrolled HR-PCD upon pathogen infection, indicating that autophagy functions to restrict HR-PCD to the infection site (Liu et al. 2005). Autophagy is a conserved process for the recycling of damaged cytosolic components such as protein, lipid, and organelles and an adaptation response to nutrient starvation, developmental senescence, and pathogen attack (Thompson and Vierstra 2005; Patel et al. 2006; Klionsky et al. 2008; Kwon and Park 2008). Genetic and molecular analyses of autophagy have revealed 30 *ATG* genes in yeasts and many orthologs in other eukaryotes including *Arabidopsis* (Thompson and Vierstra 2005; Xie and Klionsky 2007). ATG proteins are key players in the autophagic pathway that consists of several consecutive stages, such as target-of-rapamycin (TOR)-mediated induction, vesicle nucleation and elongation of an isolation membrane or phagophore, docking and fusion of autophagosomes with the endo/lysosomes, and vesicle breakdown and degradation. In mammalian cells, Rab7 has been implicated in the maturation of autophagosomes and required for the progression of autophagy (Gutierrez et al. 2004; Jager et al. 2004), suggesting the implication of RabG3b in autophagy. It would be valuable to examine whether RabG3b regulates HR-PCD through its function in autophagy.

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