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Molecular cloning and characterization of a rat sarcoma-like protein from brain (*Rab*) gene in sugar beet monosomic additional line M14

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Abstract

Suppression subtractive hybridization (SSH) and differential display reverse transcription PCR (DDRT-PCR) were performed in floral organs of *Beta vulgaris* monosomic addition line M14 (*BvM14*) (2n=18+1) and a control of *Beta vulgaris* L. (2n=18). One of the differential genes was designated as *BvM14* rat sarcoma-like protein from brain, i.e., *BvM14-Rab*. The full length of *BvM14-Rab* was obtained by RACE. It had 1,051bp with an open reading frame of 609bp encoding 202 amino acids (GenBank ID DQ831861). Sequence analysis showed four conversed domains of small GTP-binding proteins and five conserved sequences of Rab protein in the deduced BvM14-Rab protein, which has an 82% homology with AtRabD2a. Real-time PCR demonstrated that *BvM14-Rab* was expressed ubiquitously in roots, stems, leaves, flowers, pistal and stamen tissues with six-fold high abundance in the stems at two days after flowering. The BvM14-Rab protein was found to be localized in plasma membrane, indicating BvM14-Rab may play an important role in protein constitution and substance trafficking. The pBI121-*BvM14-Rab* expression construct was transformed into tobacco (*Nicotiana tabacum* L.) SR-1 leaves. Positive transgenic plants were identified by western blotting with BvM14-Rab polyclonal antibody. Constitutive expression of the BvM14-Rab in tobacco led to a series of abnormal phenotypes, including easily exfoliated flower, reduced height, inflorescence and seed weight, implying potential involvement of BvM14-Rab in flower development and seed formation.

Keywords: *BvM14-Rab*, constitutive expression, expression and localization, reproductive development, sugar beet monosomic addition line M14.
Abbreviations: EST-expressed sequence tag, ER-endoplasmic reticulum, COPⅡ-coat protein Ⅱ, SSH-suppression subtractive hybridization, UTR-untranslated region, DDRT-PCR-differential display reverse transcription PCR, RACE-rapid amplification of cDNA ends.

Introduction

Transport between endoplasmic reticulum (ER), Golgi apparatus, vacuole and plasma membrane controls the processes of protein secretion, localization and degradation, which are associated with cell growth, differentiation, and organogenesis (Vernoud et al., 2003). Anterograde and retrograde trafficking of transport vesicles between different endomembrane compartments and the plasma membrane in mammalian, yeast, and plant cells is mediated by a large family of rat sarcoma (RAS)-related small GTPases, Rabs, and Ypts (Stenmark et al., 2001). The Rab proteins constitute the largest and most complicated family of monomeric small GTPases, which behave as membrane-associated molecular switches in regulating budding, transport and fusion in the organization of membranes that are essential for maintaining normal cellular functions (Molendijk et al., 2004). Rab GTPases are soluble proteins of 20~25 kDa and are conserved from yeast to animals. They contain amino acid sequences necessary for nucleotide binding and GTP hydrolysis (Olkkonen et al., 1997). Each type of Rab is associated with a specific type of vesicles to ensure correct fusion (Takai et al., 2001). Rab proteins receive upstream signals and continue on to recruit different effectors to regulate intracellular transport between membrane and cytosol. Therefore, the Rab proteins are essential for the transport and fusion of proteins and membranes through the endomembrane system to their destinations. The *Arabidopsis thaliana* genome has fifty-seven loci encoding Rab GTPases (Rutherford et al., 2002), more than the number in yeast but similar to that in humans. The AtRab GTPases can be grouped into eight subfamilies (AtRabA to AtRabH) and can be further divided into 18 subclasses based on their sequence similarity and phylogenetic clustering with yeast and mammalian orthologs. The functions of Rab GTPase family have been extensively studied in yeast and mammalian systems, but relatively few of the plant Rab orthologs have been functionally characterized (Peng et al, 2011). In the absence of effective *in vivo* trafficking assays, information on plant Rab GTPase functions has been derived from complementation and expression studies in yeast (Ueda et al., 2000) and from expression and sub-cellular localization studies using fluorescent proteins (Ueda et al., 2001; Cheung et al., 2002; Schiene et al., 2004). Vesicle trafficking

regulated by Rab proteins not only maintain the balance of endomembrane systems, but also affect diverse biological activities and functions, such as geotactic growth, cytokinesis, hormone polar transport, stomatal movement, and disease resistance (Rohrig et al., 2004). Different Rabs are localized on distinct vesicles and organelles, and the function of each Rab protein depends on its localization (Chavrier et al., 1999). GTPase is important in the secretory pathway between the ER and Golgi bodies in tobacco elongating pollen tubes and may be specialized to support the high secretory demand in these tip growing cells (Cheung et al., 2002). Rab1 isoforms are highly conserved in eukaryotes, and they have been shown to act in the early stage of biosynthetic trafficking to or through the Golgi complex (Alvarez et al., 2003; Zheng et al., 2005). Animal Rab1 and its yeast homolog Ypt1p interact with similar effector proteins to promote the targeting of *COPII* vesicles to the *cis*-Golgi (Beard et al., 2005; Grosshans et al., 2006; Cai et al., 2008). In plants, a large number of *Rab1*-related genes have been cloned and some functional studies have been reported. By sequence comparisons, *Rab1* genes can be divided into two distinct clades, *Rab-D1* and *Rab-D2* (Pereira-Lea et al., 2001; Rutherford et al., 2002; Vernoud et al., 2003). In *Arabidopsis*, there are four *RabD*-related genes, *AtRabD1* (*At3g11730*), *AtRabD2a* (*At1g02130, AtRab1b*), *AtRabD2b* (*At5g47200, AtRab1a*) and *AtRabD2c* (*At4g17530, AtRab1c*) (Vernoud et al., 2003). *AtRabD2b* and *AtRabD2c* play important roles in pollen development, germination and tube elongation. Studies with dominant-negative mutants and loss of function mutants revealed that *AtRabD2b* and *AtRabD2c* have partially redundant roles in vesicle trafficking during pollen tube growth (Peng et al, 2011). However, the *Rab-D1* subclass genes in higher plants have not been investigated.

Sugar beet monosomic addition line M14 carrying a chromosome from *Beta corolliflora* Zoss was the progeny from hybridization between a cultivated species *B. vulgaris* L. and the wild species *B. corolliflora*, which has the superior characteristics of apomixes and tolerance to disease and cold (Guo et al., 1994; Guo et al., 2001a; Guo et al., 2001b) The M14 line has been characterized with traits of diplosporic reproduction and stress tolerance (Guo et al.,2001a; Desel et al., 2002; Ge et al., 2007). To identify differentially expressed genes in M14 line, a subtractive cDNA library was prepared by SSH between the floral organs of M14 (2n=18+1) and *B.vulgaris* (2n=18) (Ma et al., 2011). One of the differentially expressed genes was found to be Rab1-related GTP binding protein (*BvM14-Rab*, GenBank ID DQ831861). Here we report the cloning and characterization of a full-length cDNA encoding this *BvM14-Rab*. To our knowledge, this is the first report on the expression pattern and function of a Rab GTPase in apomictic materials. The findings have shed light on potential mechanisms of apomixes and stress tolerance, and will contribute to our effort in maintaining hydrid vigor in crops through apomictic reproduction.

Results

Molecular cloning of BvM14-Rab cDNA sequence

Our suppression subtractive hybridization (SSH) experiments, designed to identify differentially expressed genes in floral organs of *B.vulgaris* and sugar beet M14 line, led to the discovery of a 385 bp fragment encoding a putative GTP-binding protein named BvM14-Rab. RACE experiments were done to isolate the 5′ and 3′ end of the transcript and a 1,051 bp cDNA fragment was amplified (Fig. 1A). It contained a 159 bp 5′-untranslated region (UTR), a

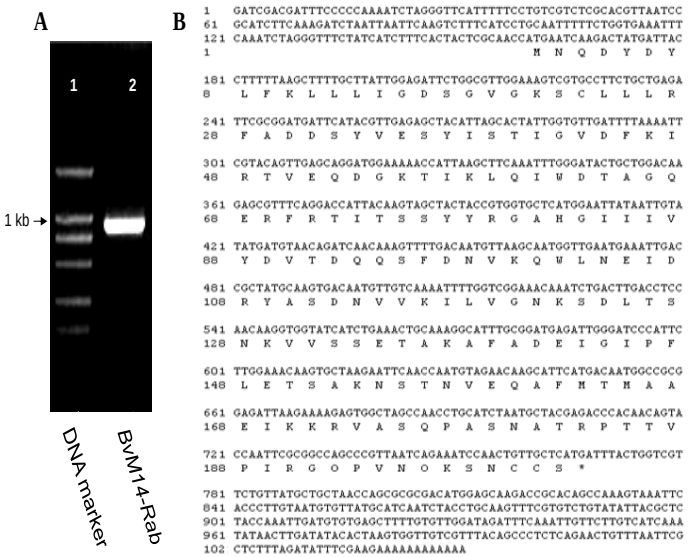

Fig 1. Verification of full length of *BvM14-Rab* from sugar beet M14 line and the deduced amino acids. (A) Electrophoresis verification of full length cDNA of *BvM14-Rab* gene. (B) The cDNA and deduced amino acid sequences of BvM14-Rab.

609 bp of coding region, and a 283 bp 3′-UTR including a consensus polyadenylation signal and a 12-bp poly(A) tail (Genbank ID: DQ831861). The deduced amino acid sequence revealed a protein consisting of 202 amino acid residues with a predicted mass of 22.44 kDa and an isoelectric point of 5.98 (Fig. 1B). To verify the SSH result of high *BvM14-Rab* expression in the M14 line, Northern blotting was conducted to show that *BvM14-Rab* was expressed at higher levels in the M14 line than the cultivated species *B. vulgaris* L. (Supplemental Fig. 1).

Sequence analysis of BvM14-Rab

BvM14-Rab polypeptide aligned well with other Rab GTP-binding proteins (Fig. 2). It was extremely close to a *Cicer arietinum* GTP-binding protein related to Rab1 (94% similarity and 87% identity). BvM14-Rab protein contains four conversed motifs of the GTPase Ras superfamily involved in GTP/Mg^{2+} binding and effector interaction (G1 to G5 in Fig.2). Further analysis of the deduced amino acid sequence revealed that BvM14-Rab contains the Rab-specific conserved region (YYRGA), the double cysteine motif (NCCS) in the C-terminal, target of the post-translational prenylation required for membrane association, and five short stretches (RabF1 to RabF5) characteristics of the Rab family of GTP-binding proteins (Pereira-Leal and Seabra, 2000, 2001). In *Arabidopsis*, the Rab GTPase family can be divided into eight subfamilies (RabA to RabH) based on sequence similarity and segregation from the yeast and mammalian orthologs (Vernoud et al., 2003). Through phylogenetic analysis, the BvM14-Rab protein was found to be clustered with *Arabidopsis* subfamily AtRabD (Supplemental Fig. 2), which regulates membrane trafficking in mammals (Tisdale et al., 1992).

Tissue specific expression of BvM14-Rab

The expression levels of *BvM14-Rab* in different plant organs and tissues including root, stem, leaf, flower, pistil and

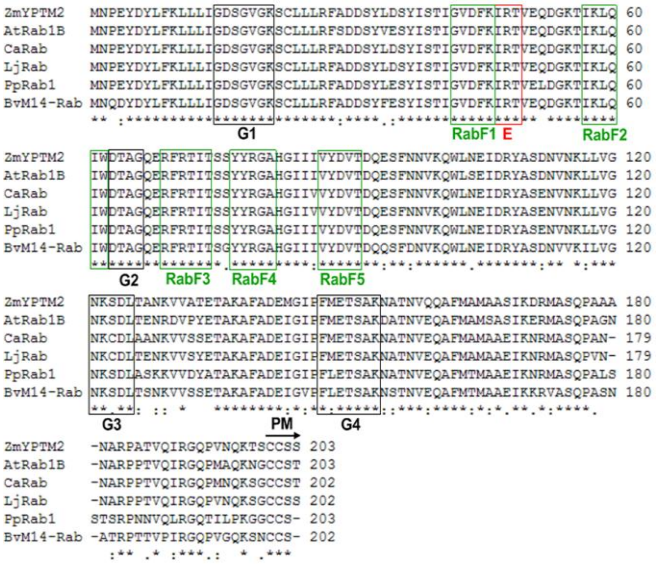

Fig 2. Multiple sequence alignment of the BvM14-Rab sequence with other reported plant GTP-binding proteins. The sequences shown here include ZmYPTM2 (*Zea mays*, gi|162458854|), PpRabA (*Poa pratensis*, gi|51468996|), AtRab1b (*Arabidopsis lyrata* subsp. gi|297842904|), CaRab (*Cicer arietinum*, gi|4586580|), LjRab1c (*Lotus japonicus*, gi|1370166|), BvM14-Rab (sugar beet M14 line). Asterisks and colons indicate identical and similar amino acids, respectively. Conserved regions of small GTP-binding protein superfamily (G1–G4) are indicated in black boxes. The unique conserved domains (RabF1-F5) are shown in green boxes. The effector region is shown in a red box, and the prenylation motif (PM) is marked by a black arrow.

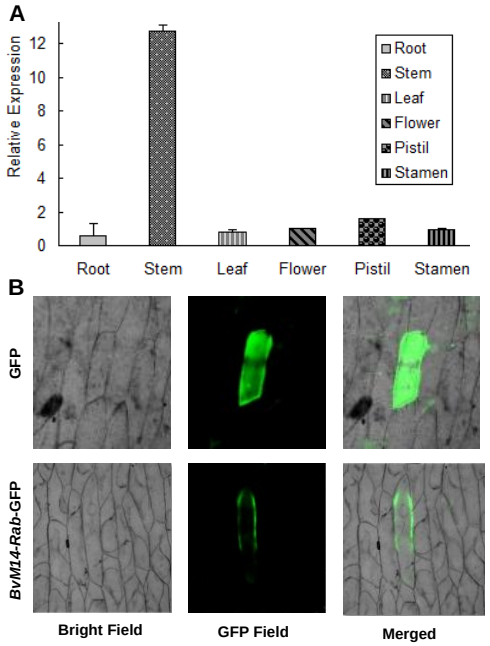

Fig 3. Tissue specific expression of *BvM14-Rab* and subcellular localization of the protein. (A) *BvM14-Rab* transcript levels in different organs and tissues of sugar beet M14 line. (B) Images showing cells expressing GFP (upper panel) and BvM14-Rab:GFP fusion protein (lower panel) examined under bright-field illumination (left), fluorescent-field illumination (middle, 488 nm) and overlay of the bright-field and fluorescent images.

stamen were analyzed (Fig. 3A). *BvM14-Rab* exhibited six-fold high expression in the M14 stem. Low levels of expression were found in other tissues and organs. These results showed that *BvM14-Rab* is expressed ubiquitously in most tissues and organs in M14 line, indicating its fundamental role in the vesicle trafficking.

Subcellular localization of BvM14-Rab

In mammals, different AtRabD orthologs are localized to the ER, ER-Golgi intermediate compartment, or Golgi compartments. In plants, AtRabD2a are associated with the Golgi apparatus and trans-Golgi network (Batoko et al., 2000; Zheng et al., 2005; Pinheiro et al., 2009). Based on these results, we predicted that BvM14-Rab would have similar localization. To our surprise, BvM14-Rab-GFP was localized to plasma membrane, while the signal of 35S::GFP was spread throughout the interior of the cell (Fig. 3B). Although phylogenetic analysis showed that BvM14-Rab has high homology with AtRabD2a, the function of BvM14-Rab in the M14 line may vary.

Ectopic expression of BvM14-Rab in tobacco

To investigate the functional significance of the *BvM14-Rab*, it was over-expressed in tobacco plants using a transgenic approach. Western blot analysis of the transgenic tobacco plants transformed with *BvM14-Rab* showed higher levels of Rab protein signal than wild-type plants (Fig. 4). This confirmed successful transformation and ectopic expression of *BvM14-Rab*. Phenotypic alterations of the transgenic plants were analyzed in T_2 generations. Fifteen independent transgenic tobacco lines were selected. The transgenic plants exhibited a series of abnormal phenotypes, including geotropism, reduced height, more shoot branching, and decreased inflorescence and fertility. For example, the transgenic plants exhibited significantly reduced height (36.2±4.5 versus 49.6±2.3 of vector control and 50.2±1.9 of wild type). They grew slowly during the vegetative growth as well as reproductive growth (Fig. 4). The inflorescences of transgenic plants were reduced compared to wild-type and empty vector plants (Fig. 4). The flowers of transgenic plants fell off when growing, and the capsules were smaller and shorter than wild-type and empty vector plants (Fig. 5). The seed yield of the transformed tobacco was significantly reduced. The capsule length and the fruit weight of transgenic tobacco were significantly decreased (Fig. 5). Rab proteins constitute the largest and most complicated family of small GTPases, which are essential for maintaining normal cellular functions. In this paper, we cloned and characterized a *rab* gene from sugar beet M14 line (*BvM14-Rab*). Though BvM14-Rab was clustered with AtRabD2a functioning in pollen development, germination and tube elongation, its location in plasma membrane is distinct from AtRabD2a in Golgi apparatus and trans-Golgi network and implies different functions of this protein. Constitutive expression of *BvM14-Rab* in tobacco has changed the flower structure characteristics, seed size, and vitality, indicating that *BvM14-Rab* is potentially involved in reproductive process and seed formation.

Discussion

Membrane trafficking in eukaryotes is dependent on accurate targeting of transport vesicles between membrane-bound compartments along endocytic pathways. Small GTPases contribute to the specification of membrane identity, accuracy

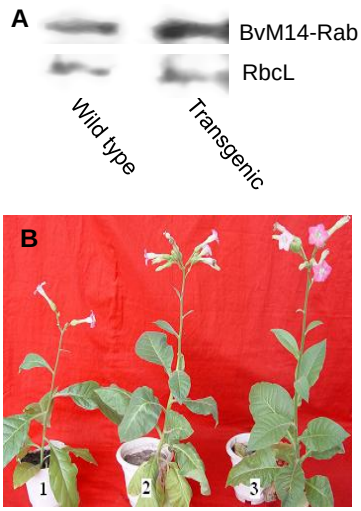

Fig 4. Phenotype of *BvM14-Rab* transgenic tobacco. (A) Western blotting shows the over-expression of BvM14-Rab in the transgenic tobacco. RbcL was used as a loading control. (B) Height and inflorescence phenotype of a transgenic plant (1) compared with vector control (2) and wild type tobacco (3).

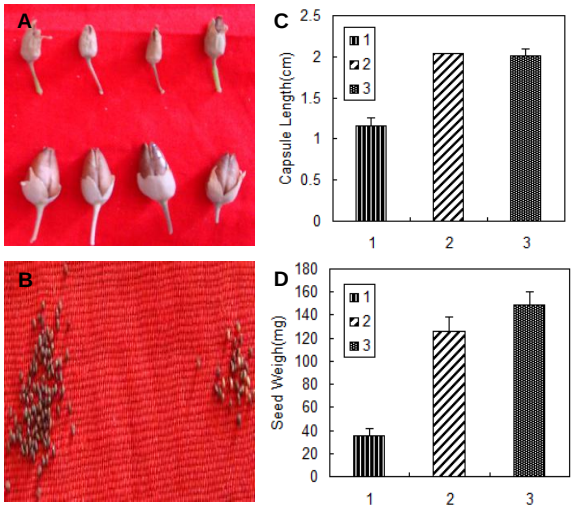

Fig 5. Capsule and seed phenotypes of BvM14-Rab transgenic tobacco. (A) Capsules of BvM14-Rab transgenic tobacco (top panel) and wild type plant (bottom panel). (B) Seeds of the wild type (left) and transgenic plant (right). (C) Capsule length of transgenic tobacco (1), empty vector (2) and wild type plants (3). (D) Seed weight of the materials in (C).

of vesicle targeting, and the recruitment of molecular motors to membranes (Behnia and Munro, 2005; Grosshans et al., 2006; Markgraf et al., 2007). Here we have isolated and characterized a cDNA encoded *BvM14-Rab* from the sugar beet M14 line. It is a novel gene with significant sequence similarity to GTP-binding proteins in the Rab family (Fig.2). BvM14-Rab was clustered with RabD2 of the RabD subclass proteins (Fig3). It has been reported that AtRabD2b and AtRabD2c participate in pollen tube elongation (Peng et al., 2011). AtRabD2b is related to the leaf senescence and ABA signaling (Peng et al., 2011). It was reported RabD2a acts in ER-Golgi traffic via different interactors (Hazel et al., 2009). Interestingly, BvM14-Rab had all the conserved domains characteristic of the Ras GTPases superfamily (Bourne et al., 1991). The G1-G5 conserved sequence motifs are required for guanine nucleotide binding and GTP hydrolysis. The hypervariable region composed of the C-terminal has been predicted to be involved in targeting Rab proteins to specific endo- and exocytic compartments. In this region, we found a conserved CCXX sequence important for post-translational processing at the C terminus; this results in addition of geranyl-geranyl lipids. Furthermore, BvM14-Rab contains the F1-F5 regions that distinguish Rabs from other small GTPases (Pereira-Leal and Seabra, 2000, 2001). Therefore, we confirmed that the BvM14-Rab is a functional Rab-related GTP binding protein. *BvM14-Rab* expression was detected in all the organs and tissues used in this study, indicating that *BvM14-Rab* is important for plant functions. Transportation of different substances through endocytosis and polar secretion may explain the differential expression of *BvM14-Rab*. For example, high levels of *BvM14-Rab* expression in M14 stems may indicate active transport activities in the stems, where BvM14-Rab plays an important role in regulating endocytosis. Rab subfamily proteins are known to be involved in the polar distribution of plant hormones through participating in vesicle transport. It has been reported that perturbation of the expression of Rgp1 in the *Rab* subfamily resulted in dwarf tobacco plants due to hormone imbalance (Kamada et al., 1992). It has also been observed that the over expression of *Rgp1* in transgenic plants caused increased cytokinin levels (Sano et al., 1994). In addition, *Arabidopsis Rha1* in the *Rab5* family plays an important role in auxin signal transduction and regulates plant organ development (Qi et al., 2005). Overexpression of *BvM14-Rab* in tobacco has changed the phenotypes of plant height, branching, flower structure characteristics, seed size, and vitality. These results indicate that *BvM14-Rab* is involved in plant growth and development, especially in cell differentiation and the reproductive development. It has been reported that auxin regulates division and differentiation of cells, affects geotropism and branching, as well as controls apical dominance and plant height (Friml, 2003; Blakeslee et al., 2005). Cytokinin is known to affect fruit development, flower abortion, and shattering. Therefore, it is reasonable to hypothesize that the phenotypic abnormalities of the tobacco expressing *BvM14-Rab* might be related to the changes of cellular hormone levels. Future research on hormone levels will be done in due course. It is believed that the action of apomixis genes in sugar beet is partly dominant rather than recessive, and the trait is controlled by multiple genes (Jassem, 1990). Transposon tagging and T-DNA mutation were carried out in *Hieracium* and two mutants with dysfunctional apomixis were isolated. The mutants were able to carry out sexual propagation (Bicknell et al., 2001). Therefore, we can speculate that the apomixis may be the result of the mutation or is affected by other factors/genes in the course of sexual propagation. In this study, the dysfunctional phenotype caused by overexpression of *BvM14-Rab* in tobacco suggests its potential function in reproduction and possible involvement in apomixes. Our results and future functional characterization studies will contribute to our goal toward maintaining hydrid vigor in crops through apomictic reproduction.

Materials and methods

Materials and growth condition

Cultivated *B. vulgaris* L. var Saccharifera Alef (2n=18) is a commercial germplasm that is widely used for sugar industry. Line M14 is a set of monosomic addition lines with the No.9 chromosome of *Beta corolliflora Zoss.* It displays characteristics of apomixes and tolerance to disease and cold. Apomixes can be used to maintain heterosis. Therefore, *B. vulgaris* and the monosomic addition line M14 (containing the 18 chromosomes of *B. vulgaris* and the 9th chromosome of *B. corolliflora*, 2n=18+1) (Guo et al., 2001a; Guo et al., 2001b) were chosen as experimental materials and grown in the greenhouse of Heilongjiang University, Harbin, China. The organs and tissues (root, stem, leaf, flower, pistil and stamen) at two days after flowering in M14 and *B. vulgaris* were immediately frozen in liquid nitrogen and stored at –80 ^{o}C until further analysis.

Molecular cloning of the BvM14-Rab gene

A cDNA library was obtained through SSH using mRNA from the floral organs of monosomic additional line M14 as tester and from *B.vulgaris* as driver (Ma et al., 2011). An EST clone (Me-263) identified through random sequencing from the library showed maximum similarity to a Rab family GTPase from *Cicer arietinum* (BAA76422) in BLASTx analysis. The 5'-3' cDNA fragments of the EST were amplified using the SMART RACE-kit from CLONTECH. A 0.4 kb cDNA fragment was pre-amplified from mRNA, followed by extending the fragment into a full-length cDNA, *BvM14-Rab*.

Sequence analysis of BvM14-Rab

Protein targeting prediction was done using Target P 1.1 (Nielsen et al., 1997; Emanuelsson et al., 2000). HMMTOP was used for putative transmembrane prediction (Tusna´et al., 1998; Tusna et al., 2001). Alignment of protein sequences was performed using CLUSTALW with default parameters (Higgins et al., 1994). Signal peptide analysis was performed using the Signal P 3.0 program. Phylogram tree of *BvM14-Rab* was generated using ClustalX (ver 1.8) using a Phylip distance matrix with 1000 bootstrap trials and MEGA4 software (Thompson et al., 1994).

Real-time quantitative PCR analysis

Total RNA was extracted from different tissues and organs of M14 using RNeasy extraction kit (Qiagen, CA, USA) and treated with RNAase-free DNAase I according to the manufacturer's instructions. First-strand cDNA was synthesized using total RNAs by priming with an oligo-dT-anchor at 42 ^{o}C for 30 min using a Superscript-II Reverse Transcriptase (TAKARA Inc.) according to the manufacturer's instructions.

Quantitative PCR was conducted with SYBR®Green PCR Mix and gene-specific primers (5'-CAATGTTAAGCAATGGTTGAATGAA-3' and 5'-ATACTGTTGTG GGTCTCGTAGCATT-3'). The 18S rRNA was chosen as loading control (Rajabi et al., 2007). Each PCR reaction (20µl final volume) contained 2µl of template cDNA, 0.8µl of each primer and 1×SYBR® Green PCR Master Mix. Thermo-cycling conditions were: an initial enzyme activation of 20 second at 95 ^{o}C, followed by 40 cycles of denaturation for 10 s at 94 ^{o}C, annealing for 30 s at 58 ^{o}C and extension for 30 s at 72 ^{o}C, with a final melt gradient starting from 55 ^{o}C and heating to 95 ^{o}C at a rate of 0.06 ^{o}C s^{-1}. The real-time PCR reactions were carried out in a chromo 4 Bio-rad Real Time PCR System. Fluorescence was measured at the 497 nm (excitation) and 521 nm (detection) wavelengths at the end of each extension step and at each 1 ^{o}C increment of the melt profile. Primer specificity was confirmed by analyzing dissociation curves of the PCR amplification products. Each DNA sample was quantitatively analyzed three times.

Subcellular localization analysis of BvM14-Rab

For subcellular localization analysis, BvM14-Rab protein fused to GFP was used to bombard onion epidermal cells as described (Liang et al. 2010). BvM14-Rab signal was visualized using an inverted LSM 5 CLSM microscope (Zeiss Inc., Germany). Excitation wavelengths of 488 nm and 560 nm were used for GFP and FM4-64 emission, respectively. Images were captured and analyzed using LSM 5 Pascal software (Zeiss Inc., Germany).

Expression of BvM14-Rab in E. coli and antibody preparation

The coding region of *BvM14-Rab* was amplified using PCR, with primers (5'-GCGGAGCTCCATCTTTCACTACTCGCAACC-3' and 5'-GCGCTCGAGGCT CACAC ATCAATTTGGTAG -3') designed to create a *Sac* I site at 5' end and *Xho* I site (underlined) at the 3' end. The amplified cDNA was subcloned into expression vector pET28a at *Sac* I/*Xho* I sites (Novagen Inc., USA), resulting in the plasmid pET28a-*BvM14-Rab*. The construct was introduced into *E. coli* [BL21 (DE3)] cells for protein expression. The *E. coli* cells carrying pET28a-*BvM14-Rab* and control vectors were grown at 37°C in LB medium containing 100 µg ml $^{-1}$ kanamycin to an OD_{600} of 0.5, and were induced with 1.0 mM IPTG. Cells were harvested after 6 h and the protein profile was analyzed using 15% SDS–PAGE. Total protein was collected and purified using a HisBind purification kit (Novagen Inc., USA). Purified BvM14-Rab protein was used for antibody production in New Zealand white rabbits.

Generation of BvM14-Rab transgenic plants

BvM14-Rab was amplified using primers: 5'-CGCTCTAGACATC TTTCACTACTCGCAACC-3' and 5'-TCCCCCGGGCGTAATATACAGACACG AAAC-3'. The amplified product was digested with *Xba* I and *Xma* I and ligated to pBI121 to construct the expression vector pBI121-*BvM14-Rab*. In the recombinant plasmid, the *rab* and reporter *uidA* genes were under the control of separate CaMV *35S* promoter and *NOS* polyadenylation signals. The plasmid was transformed into *Agrobacterium tumefaciens* EHA105, and then into tobacco (*Nicotiana tabacum* L) SR-1 following a leaf disc transformation procedure (Horsch et al., 1985). Putative transgenic plants were selected using hygromycin and screened using a histochemical GUS assay (Jefferson, 1987). The positive transgenic plants were then identified with genomic and transcriptive PCR.

Western Blotting

For Western blotting, protein extracts from T_2 leaves were separated on 15% polyacrylamide gels and blotted onto PVDF membranes (0.2-mm pore size; Millipore Inc.). Blots

were blocked in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (vol/vol)) containing 1% BSA (wt/vol) and incubated with anti-BvM14-Rab antiserum at a 1:1,280 dilution and anti-RbcL antibody (1:50,000), respectively. Blots were washed, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and developed by using an enhanced chemiluminescence system (Pierce Inc., USA).

Phenotype and Statistical analysis

Phenotypic alterations were analyzed in T_2 plants. Twelve independent transgenic tobacco lines were selected and geotropism, plant height, shoot branching number, inflorescence number, and seed fertility were measured, respectively. All experiments were carried out independently three times.

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