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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, COP II -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, AtRabla) and AtRabD2c (At4g17530, AtRablc) (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<sup>2+</sup> 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

AtRab1B       MNPEYDYLFKLLLISDSGVGRSCLLLRF3DDSYUSYISTIGVDFRIFTFEQDGRFLLQ       60         Carab       MNPEYDYLFKLLLISDSGVGRSCLLLRF3DDSYLDSYISTIGVDFRIFTFEQDGRFLRLQ       60         PgRab1       MNPEYDYLFKLLLISDSGVGRSCLLLRF3DDSYLDSYISTIGVDFRIFTFEQDGRFLRLQ       60         bmM14-Rab       MNQUYDYLFKLLLISDSGVGRSCLLLRF3DDSYLDSYISTIGVDFRIFTFEDGGRFLRLQ       60         bmM14-Rab       MNQUYDYLFKLLLISDSGVGRSCLLLRF3DDSYLDSYISTIGVDFRIFTFEDGGRFLRLQ       60         bmM14-Rab       MNQUYDYLFKLLLISDSGVGRSCLLLRF3DDSYLESYISTIGVDFRIFTFEDGGRFLRLQ       60         carabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLVG       120         carabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLVG       120         carabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         carabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         bwM14-Rabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         carabi       IMDTAGQERFRT13SYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         bwM14-Rabi       IMDTAGQERFRT1SSYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         carabi       IMDTAGQERFRT1SSYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG       120         bwM14-Rabi       IMDTAGQERFRT1SSYYRGAHGII IVYDVTDQESFNNVKQMLNEIDRYASDNVRLLUG        <		MNPEYDY	LFKLLLIG	DSGVGKS	CLLLRFADE	SYLDSYIST	IGVDFKIRT	VEQDGKTIKLQ	60
CaRab MNPEYDYLFKLLLISDSGVGRSCLLLRFADDSYLDSYISTIGVDFRIFTFEQGGRVFLLQ 60 LJRAb MNPEYDYLFKLLISDSGVGRSCLLLRFADDSYLDSYISTIGVDFRIFTFEQGGRVFLQ 60 BVM14-Rab MNPEYDYLFKLLISDSGVGRSCLLLRFADDSYLBSYISTIGVDFRIFTFEQDGRVFLQ 60 TWFTAGD2FRFTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURDGRVFLUG 60 TWFTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURDGRVFLUG 120 CaRab INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 LJRAb INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 LJRAb INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 LJRAb INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 FPRab1 INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 FM14-Rab INDTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 TMFTAGD2FFRTISSYYRGAHGIIVYDVTDQDSYNNYGVLNEIDFYRSTURNLUG 120 FM14-Rab INDTAGD2FFRTISSYYRGAHGIIFYDVTTDQDSYNNYGVLNEIDFYRSTURNLUG 120 TMFTAGD2FFRTISSYYRGAHGIIFYDVTTDQQSFNNYGVLNEIDFYRSTURNLUG 120 FM14-Rab INDTAGD2FFRTISSYYRGAHGIIFYDVTTDQQSFNNYGVLNEIDFYRSTURNLUG 120 FM14-Rab INSDIJFANKVVATETARFADEIGIFFMETSAKNATNVQQAFMAMAASIRDFNASQFAA 180 NKSDIJFANKVVSSTAFAFADEIGIFFMETSAKNATNVQQAFMAMASIRDFNASQFAA 180 NKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMASIRDFNASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMABEINNMASQFAA 180 HKSDIJFENNVGIRGQFVQRGSCCS 203 CARAB NARPPTVQIRGQPVQRGSCCS 203 CARAB NARPPTVQIRGQPVQRGSCCS 202 PDRAB1 NARPPTQIRGQPVQRGSCCS 203 CARA	AtRab1B	MNPEYDY	LFKLLLIG	DSGVGKS	CLLLRFSDD	SYVESYIST	IGVDFKIRT	VEQDGKTIKLQ	60
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ZmyPM2         INDTAGD2EFFFTI3SYYRGABGIIIVYDVTDQ2SFNNVKQMLNEIDFXASDWVRLLUG         120           AtRab1B         INDTAGD2EFFFTI3SYYRGABGIIVYDVTDQ2SFNNVKQMLNEIDFXASDWVRLLUG         120           Likbradp2EFFTI3SYYRGABGIIVYDVTDQ2SFNNVKQMLNEIDFXASEWVRLLUG         120           Ljkab         INDTAGD2EFFTI3SYYRGABGIIVYDVTDQ2SFNNVKQMLNEIDFXASEWVRLLUG         120           PgRab1         INDTAGD2EFFTI3SYYRGABGIIVYDVTDQ2SFNNVKQMLNEIDFXASEWVRLLUG         120           BVM14-Rab         INDTAGD2EFFTI3SYYRGABGIIVYDVTDQ2SFNNVKQMLNEIDFXASEWVRLLUG         120           CG2         RabF3         RabF4         RabF4           C3         RabF4         RabF5         RabF7           NKSDLTENKVVSYETAKAFADEIGIFFMETSAKNATNVEQAFMAMASIKDFNASQPAAN         180           NKSDLTENKVVSYETAKAFADEIGIFFMETSAKNATNVEQAFMAMASIKDFNASQPAAN         180           NKSDLTENKVVSYETAKAFADEIGIFFMETSAKNATNVEQAFMAMASIKDFNASQPAN         180           NKSDLTENKVVSYETAKAFADEIGIFFMETSAKNATNVEQAFMAMAZEIKNFMASQPAN         179           NKSDLTENKVVSYETAKAFADEIGIFFMETSAKNATNVEQAFMAMAZEIKNFMASQPAN         180           W14-Rab         NKSDLTENKVVSYETAKAFADEIGIFFETETSAKNATNVEQAFMAMAZEIKNFMASQPAN         180           W14-Rab         NKSDLTENKVVSSETAKAFADEIGIFFETETSAKNATNVEQAFMTMAAEIKNFMASQPAN         180           W14-Rab         NKSDLTENKVVSSETAKAFADEIGIFFETETSAKNATNVEQAFMTMAAEIKNFMASQPAN         180 <td></td> <td></td> <td></td> <td>G1</td> <td></td> <td></td> <td>RabF1 E</td> <td>RabF</td> <td>2</td>				G1			RabF1 E	RabF	2
AcRabIB         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLSEIDRYASDUVRLLUG         120           CaRab         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLEIDRYASDUVRLLUG         120           IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLEIDRYASDUVRLLUG         120           PpRab1         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLEIDRYASDUVRLLUG         120           BvM14-Rab         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLNEIDRYASDUVRLLUG         120           SmY14-Rab         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQESFNNVKQMLNEIDRYASDUVVKLLUG         120           SmY14-Rab         IMDTAGQEFFRTISSYYRGAHGIIUVIDUTDQSFDNVKQMLNEIDRYASDUVVKLLUG         120           SmYPT2         NKSDIPANKVVATETAKAFADEMGI FMETSAKNATNVQQAFMAMAASIKDFMASQPAA         180           CaRab         NKSDIPANKVVATETAKAFADEIGI FMETSAKNATNVEQAFMAMASIKDFMASQPAA         180           NKSDIPANKVVSSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNNASQPAA         180           NKSDIPSKVVOSSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNMASQPAS         180           NKSDIPSKVVOSSETAKAFADEIGI FMETSAKNSNTNVEQAFMAMAEIKNMASQPAS         180           NKSDIPSKVVOSSETAKAFADEIGI FMETSAKNSTNVEQAFMAMAEIKNMASQPAS         180           MM14-Rab         NARPHYQIRGQPVNQKTSCCSS         203           CaRab         NARPFYQIRGQPMNQKSCCSS         203           CaRab         NARPFYQIRGQPUNQKSCCSS         202                  NARP	ZmYPTM2	IWDTAGQ	RFRTITS	SYYRGAN	GIIIVYDVI	DQESFNNVK	QWLNEIDRY	ASDNVNKLLVG	120
CaRab IMDTAGQERFRTISSYYRGAHGIIVVIDVTDQESFNNVKQMLNEIDRYASENVNKLUG 120 JGRAD IMDTAGQERFRTISSYYRGAHGIIVVIDVTDQESFNNVKQMLNEIDRYASENVNKLUG 120 JFRADI IMDTAGQERFRTISSYYRGAHGIIVVIDVTDQESFNNVKQMLNEIDRYASENVNKLUG 120 BVM14-Rab IMDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVTDQSFDNVKQMLNEIDRYASENVNKLUG 120 LINDTAGQERFRTISSYYRGAHGIIIVVIDVEQAFMAMAASIKDRMASQFAAN 180 NKSDLTENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMAAEIKNMASQFANN 179 NKSDLTENNVVSSTAFAFADEIGIFFMETSAKNATNVEQAFMAMAAEIKNMASQFANN 179 NKSDLTENNVVSSTAFAFADEIGIFFLETSAKNATNVEQAFMAMAAEIKNMASQFANN 180 <u>t+++</u> ; : + ++++++; ++++++; ++++++; ++++++; ++++++	AtRab1B	IWDTAGQ	ERFRTITS	SYYRGAN	GIIIVYDVI	DQESFNNVK	QWLSEIDRY	ASDNVNKLLVG	120
Ljab INDTAGDZFFRTIJSSYTRAAGIIUVTVDVDCSFNNVKQNLNEIDRIASUVNKLUG 120 PgRab1 INDTAGDZFFRTIJSSYTRAAGIIUVTVDVDCSFNNVKQNLNEIDRIASUVNKLUG 120 BWM14-Rab INDTAGDZFFRTIJSSYTRAAGIIUVVDVDCQSFNNVKQNLNEIDRIASUNVKLUG 120 CC RabF3 RabF3 RabF4 RabF54 ACRab1 NKSDUPANKVVATETAKAFADEMGI FMETSAKNATNVQQAFMAMAASIKDRMASQPAAA 180 NKSDUPANKVVATETAKAFADEMGI FMETSAKNATNVQQAFMAMAASIKDRMASQPAAA 180 NKSDUPENNDVFYETAKAFADEIGI FMETSAKNATNVQQAFMAMAASIKDRMASQPAAA 180 NKSDUPENNDVFYETAKAFADEIGI FMETSAKNATNVQQAFMAMAASIKDRMASQPAAA 180 NKSDUPENNVVSSTAKAFADEIGI FMETSAKNATNVEQAFMAMAAZIKNMASQPAA 179 NKSDUPANKVVSSTAKAFADEIGI FMETSAKNATNVEQAFMAMAAZIKNMASQPAN 179 NKSDUPANKVVSSTAKAFADEIGI FMETSAKNATNVEQAFMAMAAZIKNMASQPAN 179 NKSDUPANKVVSSTAKAFADEIGI FMETSAKNATNVEQAFMAMAZIKNMASQPAN 180 ****;;;******************************	CaRab	IWDTAGQ	ERFRTITS	SYYRGAN	GIIVVYDVI	DQESFNNVK	QWLNEIDRY	ASENVNKLLVG	120
PpRabl         Imptadgesprerigsiyrscahgdilivvovmpogessmnvkgulneidersakunkluggi         120           Bvml4-Rab         Imptadgesprerigsiyrscahgdilivvovmpogessmnvkgulneidersakunkluggi         120           G2         RabF3         RabF4         RabF5           ZmyPTM2         NKSDITANKVVATETAKAFADEMGI FMETSAKNATNVQQAFMAMAASIKDRMASQFAAN         180           CaRab         NKSDITANKVVATETAKAFADEMGI FMETSAKNATNVQQAFMAMAASIKDRMASQFAAN         180           CaRab         NKSDITANKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAASIKDRMASQFAAN         179           LjRab         NKSDITENKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITENKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITENKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAEIKNRMASQFAN-         179           NKSDITSNKVVSETAKAFADEIGI FMETSAKNATNVEQAFMAMAEIKNKASQFAN-         180           MYTM2	LjRab	IWDTAGQ	ERFRTITS	SYYRGAN	GIIVVYDVI	DQESFNNVK	QWLNEIDRY	ASENVNKLLVG	120
BwM14-Rab       Imptradgesprerigsdyrgeahealtivvvvtugogsenvrkoulneidersaturvitug 120         SzmyPTM2       RabF3       RabF4       RabF4       RabF5         ZmyPTM2       NKSDIENNKVVATETAKAFADENGI FFKETSAKNATNVQQAFMAMAASIKDRMASQPAAN 180         NKSDIENNKVVASETAKAFADEIGI FFKETSAKNATNVQQAFMAMAASIKDRMASQPAAN 180         NKSDIENNKVVSSETAKAFADEIGI FFKETSAKNATNVEQAFMAMAASIKDRMASQPAAN 180         NKCDIANNKVSSETAKAFADEIGI FFKETSAKNATNVEQAFMAMAASIKDRMASQPAN 179         NKCDIANNKVSSETAKAFADEIGI FFKETSAKNATNVEQAFMAMAAEIKNNMASQPAN 179         NKCDIANNKVSSETAKAFADEIGI FFKETSAKNATNVEQAFMAMAAEIKNNMASQPAN 179         NKSDIENNKVSSETAKAFADEIGI FFLETSAKNATNVEQAFMAMAAEIKNMASQPAN 180         SmyPTM2       -NARPATVQIRGQPVNQKTSCCSS 203         AcRab1B       -NARPPTVQIRGQPMAQKSCCST 203         AcRab1       -NARPPTVQIRGQPVNQKSCCSS 202         PRAB1       STSFPNNVQLRGQTILFRGCCS- 203         STSFPNNVQLRGQTILFRGCCS- 203       203	PpRab1	IWDTAGQ	ERFRTITS	SYYRGAN	GIIIVYDVI	DQESFNNVK	QWLNEIDRY	ASENVNKLLVG	120
Laberty descendent         Litter in the intervention of the interventinterventeq intervention of the interventinte intervention of th	BvM14-Rab	IWDTAGQ	ERFRTITS	GYYRGAH	GIIIVYDVI	DQQSFDNVK	QWLNEIDRY	ASDNVVKILVG	120
G2         Kabba         Rabba           Caryptm2         NKSDIJANKVVAJETAKAFADEMGI EMETSAKNATNVQQAFMAMAASIKDEMASQFAAA         180           ArRabiB         NKSDIJENNDVYAJETAKAFADEMGI EMETSAKNATNVQQAFMAMAASIKDEMASQFAAA         180           Carab         NKSDIJENNDVYSETAKAFADENGI EMETSAKNATNVQQAFMAMAASIKDEMASQFAAA         180           LjRab         NKSDIJENNDVYSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNMASQFAAA         179           LjRab         NKSDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNMASQFAAA         179           NKSDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNMASQFAAA         180           NKSDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNASQFAAA         180           NKSDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNASQFAAA         180           VASDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNASQFAAA         180           VASDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAAEIKNNASQFAAA         180           VASDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAEIKNASQFAAA         180           VASDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAEIKNNASQFAAA         180           VASDIJENNVVSSETAKAFADENGI EMETSAKNATNVEQAFMAMAEIKNNASQFAAA         180           VASDIJENNVVSSETAKAFADENNVSQAFA         203           ArabiB         -NARPPTVQIRGQPMQKSGCSS 202           PAMADIA         -NARPPTVQIRGQPMAQKSCCSS 202                       TARDENNUFARAFANINGAGUT			*******		****	**:**:***	***.*****	**:** *:***	
$ \begin{array}{llllllllllllllllllllllllllllllllllll$		G2	RabF3	RabF4	RabE	0			
AtRab1B         NKSDLIPENRDVFYETATAFADEIGIEFMETSAKDATNVEQAFMAMSASIERENKASQFAON 180           Carab         NKCDLIPENRVVSSETAKAFADEIGIEFMETSAKNATNVEQAFMAMABEIKNRMASQFAON 179           NKCDLIPENRVVSSETAKAFADEIGIEFMETSAKNATNVEQAFMAMABEIKNRMASQFAN-179           NKCDLIPENRVVSSETAKAFADEIGIEFMETSAKNATNVEQAFMAMABEIKNRMASQFAN-179           NKSDLISKKVVDYATAFAFADEIGIEFMETSAKNATNVEQAFMTMABEIKNRMASQFAN-179           NKSDLISKKVVDYATAFAFADEIGIEFMETSAKNATNVEQAFMTMABEIKNRMASQFAN-179           SG3         PM           G3         PM           G4         - NARPHTYQIRGQPVNQKTGCCS5           ZmYPTM2         - NARPHTYQIRGQPMAQKNGCCST           - NARPHTYQIRGQPVNQKTGSCCS5         203           - NARPHTYQIRGQPUNGKSCCS5         202           PERABL         STSRPNNYQLRGQPTILPRGGC25         203           VM & DE DETERVENTER         200	m-11mm10	and on the second							
Carab         NKCDLFANKVVSSETAKAFADEIGIEFMETSAKNATNVEQAFMAMAAEIKNNMASQFNN-179           LjRab         NKCDLFENKVVSSETAKAFADEIGIEFMETSAKNATNVEQAFMAMAAEIKNNMASQFNN-179           PSRab1         NKSDLFENKVVSSETAKAFADEIGIFMETSAKNATNVEQAFMAMAAEIKNNMASQFNN-179           NKSDLFENKVVSSETAKAFADEIGIFMETSAKNATNVEQAFMAMAAEIKNNMASQFNN-179           BVM14-Rab         NKSDLFENKVVSSETAKAFADEIGUFFLETSAKNATNVEQAFMIMAAEIKNMASQFNN-179           G3         PM           G4	2mi PTMZ	NKSDUTA	NKVVATET	AKAFADE	MGIFFMETS	AKNATNVQQ	AFMAMAASI	KDRMASQPAAA	180
Ljkab         NKCDLIPENKVVSYETAKAFADEIGIEFPLETSAKNATNVEQAFMAMAAEIKNIKMASQPUN-179           PpRabl         NKSDLIASKKVVDYATAKAFADEIGIEFPLETSAKNATNVEQAFMIMAAEIKNIKMASQPAN-180           BrM14-Rab         NKSDLISNKVVSSETAKAFADEIGUFPLETSAKNATNVEQAFMIMAAEIKNIKMASQPAN-180           GG         PM           -NARPATVQIRGQPVNQKTSCCSS         203           AcrablB         -NARPPTVQIRGQPMAQKNGCCST         203           Carab         -NARPPTVQIRGQPMAQKSCCSS         202           Jjkab         -NARPPTVQIRGQPILEFGGCCS-203           STSRPNNVQLRGQTILFRGGCCS-203         203	AtRab1B	NKSDLTA	NRVVATEI	AKAFADE	MGIFFMETS	AKNATNVQQ AKDATNVEQ	AFMAMAASI AFMAMSASI	KDRMASQPAAA KERMASQPAGN	180
PpRab1         NKSDLÄSKKVVDYATATAFADEIGT FFLETSAKNATNVEQAFMTMAAEIKNRMASOPALS 180           BvM14-Rab         NKSDLTSNKVVSSETAKAFADEIGT FFLETSAKNSTNVEQAFMTMAAEIKKRVASOPALS 180           ************************************	AtRab1B CaRab	NKSDLTAI NKSDLTEI NKCDLAAI	NKVVATEI NRDVPYEI NKVVSSEI	'AKAFADE 'AKAFADE 'AKAFADE	MGIFFMETS IGIFFMETS IGIFFMETS	AKNATNVQQ AKDATNVEQ AKNATNVEQ	AFMAMAASI AFMAMSASI AFMAMAAEI	KDRMASQPAAA KERMASQPAGN KNRMASQPAN-	180 180 179
Bvm14-Rab         NKSDUPSNRVVSSETARAFADEIGVEFLETSAKNSTNVEQAFMTMAAEIKKRVASOPASN 180           **.**;         ************************************	AtRab1B CaRab LjRab	NKSDLTEI NKCDLAAI NKCDLTEI	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI	'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE	MGIFFMETS IGIFFMETS IGIFFMETS IGIFFMETS	aknatnvqq akdatnveq aknatnveq aknatnveq	AFMAMAASI AFMAMSASI AFMAMAAEI AFMAMAAEI	KDRMASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPVN-	180 180 179 179
Image: Stars         Image: Stars<	AtRab1B CaRab LjRab PpRab1	NKSDLTAI NKSDLTEI NKCDLAAI NKCDLTEI NKSDLASI	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI KKVVDYAI	'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE	MGIPFMETS IGIPFMETS IGIPFMETS IGIPFMETS IGIPFLETS	aknatnvqq akdatnveq aknatnveq aknatnveq aknatnveq	AFMAMAASI AFMAMSASI AFMAMAAEI AFMAMAAEI AFMTMAAEI	KDRMASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPVN- KNRMASQPALS	180 180 179 179 180
G3 PM G4NARPATVQIRGQPVNQKTSCOSS 203 AtRab1BNARPPTVQIRGQPMAQKNGCOST 203 CaRabNARPPTVQIRGQPMQKSGCOST 202 LjRabNARPPTVQIRGQPVNQKSGCCSS 202 PpRab1 STSRPNNVQLRGQTILPEGGCCS- 203	AtRab1B CaRab LjRab PpRab1 BvM14-Rab	NKSDLTAI NKSDLTEI NKCDLAAI NKSDLASI NKSDLASI	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI KKVVDYAI NKVVSSEI	'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE 'AKAFADE	MGIFFMETS GIFFMETS GIFFMETS GIFFMETS GIFFLETS GVFFLETS	aknatnvqq akdatnveq aknatnveq aknatnveq aknatnveq aknatnveq aknstnveq	AFMAMAASI AFMAMSASI AFMAMAAEI AFMAMAAEI AFMTMAAEI AFMTMAAEI	KDRMASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPVN- KNRMASQPALS KKRVASQPASN	180 180 179 179 180 180
ZmYPTM2         -NARPATUQIRGQPWNQKTSCCS5         203           AtRab1B         -NARPPTVQIRGQPMAQKNGCCS7         203           Carab         -NARPPTVQIRGQPSCCS7         202           LjRab         -NARPPTVQIRGQPVNQKSGCCS5         202           PpRab1         STSRPNNYQLRGQTIPRGGCCS6         203	ZMIPIMZ AtRab1B CaRab LjRab PpRab1 BvM14-Rab	NKSDLTAI NKSDLTEI NKCDLAAI NKSDLASI NKSDLASI ** **:	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI KKVVDYAI NKVVSSEI :: *	AKAFADE AKAFADE AKAFADE AKAFADE AKAFADE AKAFADE	MGIPFMETS CIGIPFMETS CIGIPFMETS CIGIPFLETS CIGVPFLETS	AKNATNVQQ AKDATNVEQ AKNATNVEQ AKNATNVEQ AKNATNVEQ AKNSTNVEQ	AFMAMAASI AFMAMSASI AFMAMAAEI AFMTMAAEI AFMTMAAEI AFMTMAAEI	KDRMASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPVN- KNRMASQPALS KKRVASQPASN	180 180 179 179 180 180
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CaRab         -NARPPTVQIRGQPMNQKSGCCST         202           LjRab         -NARPPTVQIRGQPVNQKSGCCSS         202           PpRab1         STSRPNNYQLKGQTIDFKGGCCS-         203	ZmrFFM2 AtRab1B CaRab LjRab PpRab1 BvM14-Rab ZmYPTM2	NKSDITA NKSDITE NKCDIAA NKSDIAS NKSDIAS NKSDITS ** **: G3 -NARPAT	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI KKVVDYAI NKVVSSEI :: * *	AKAFADE AKAFADE AKAFADE AKAFADE AKAFADE AKAFADE MQKTSCC	MGIEFMETS CIGIEFMETS CIGIEFMETS CIGIEFMETS CIGIEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFLETS CIGVEFMETS C	AKNATNVQQ AKDATNVEQ AKNATNVEQ AKNATNVEQ AKNATNVEQ AKNSTNVEQ **::***:*	AFMAMAASI AFMAMSASI AFMAMAAEI AFMAMAAEI AFMTMAAEI AFMTMAAEI ***:*:*.*	KDRMASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPAN- KNRMASQPALS KKRVASQPALS *.*.***	180 180 179 179 180 180
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PpRab1 STSRPNNVQLRGQTILPRGGCCS- 203	2mrPTM2 AtRab1B CaRab LjRab PpRab1 BvM14-Rab 2mYPTM2 AtRab1B CaRab	NKSDITAI NKSDITEI NKSDIAAI NKSDIASI NKSDIASI NKSDITSI ** **: G3 -NARPAT -NARPT -NARPT	NKVVATEI NRDVPYEI NKVVSSEI NKVVSYEI KKVVDYAI NKVVSSEI :: * VQIRGQPV VQIRGQPM VQIRGQPM	ARAFADE ARAFADE ARAFADE ARAFADE ARAFADE ARAFADE NQRTSCC INQRTSCC	MGIEFMETS IGIEFMETS IGIEFMETS IGIEFLETS IGUEFLETS IGVEFLETS	iaknatnvqq iakdatnveq iaknatnveq iaknatnveq iaknatnveq iaknstnveq i.:***:*	AFMAMAASI AFMAMAASI AFMAMAAEI AFMAMAAEI AFMTMAAEI AFMTMAAEI	KDRNASQPAAA KERMASQPAGN KNRMASQPAN- KNRMASQPVN- KNRMASQPALS KKRVASQPALS *.*.***	180 180 179 179 180 180
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**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<sub>2</sub> 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).

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



**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).

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 9<sup>th</sup> 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 °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 °C for 30 min using a Superscript-II Reverse Transcriptase (TAKARA Inc.) according to the manufacturer's instructions.

Quantitative PCR was conducted with SYBR<sup>®</sup>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<sup>®</sup> Green PCR Master Mix. Thermo-cycling conditions were: an initial enzyme activation of 20 second at 95 °C, followed by 40 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 58 °C and extension for 30 s at 72 °C, with a final melt gradient starting from 55 °C and heating to 95 °C at a rate of 0.06 °C s<sup>-1</sup>. 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 °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'-GCG<u>CTCGAG</u>GCT 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<sup>-1</sup> kanamycin to an OD<sub>600</sub> 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 amplified using primers: was 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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