AtBS14a and AtBS14b, two *Bet1*/*Sft1*-like SNAREs from *Arabidopsis thaliana* that complement mutations in the yeast *SFT1* gene[1]

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Abstract SNAREs are membrane-associated proteins that play a central role in vesicle targeting and intra-cellular membrane fusion reactions in eukaryotic cells. Here we describe the identification of AtBS14a and AtBS14b, putative SNAREs from *Arabidopsis thaliana* that share 60% amino acid sequence identity. Both AtBS14a and BS14b are dosage suppressors of the temperature-sensitive growth defect in *sft1-1* cells and over-expression of either AtBS14a or AtBS14b can support the growth of *sft1*Δ cells but not *bet1*Δ cells. These data together with structure–function and biochemical studies presented herein suggest that AtBS14a and AtBS14b share properties that are consistent with them being members of the Bet1/Sft1 SNARE protein family. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: SNARE; Functional complementation; *BET1*; *SFT1*; *Arabidopsis thaliana*

1. Introduction

The transport of proteins between compartments of the secretory pathway is mediated by vesicles that bud from one compartment and selectively dock and fuse with another [1]. A key event in this process is the specific recognition of the target membrane by the vesicle, a process that is necessary in order to maintain the organization of the secretory pathway. Vesicle target recognition and the membrane fusion event that follows are in part the result of the pairing of SNARE proteins (soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors) located on both the transport vesicle and target compartment [2,3]. We are interested in understanding the precise role(s) of SNAREs in traffic through the yeast Golgi [4]. Here we have used functional complementation in yeast as an assay to isolate homologues of the essential yeast Golgi SNARE, *SFT1* [5] from *Arabidopsis thaliana*.

2. Materials and methods

2.1. Strains and media

The following yeast strains were used in this study: SARY130 (*MATα sft1Δ::LEU2 leu2-1,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9* [*CEN6, TRP1, sft1-1*]), SARY270 (*MATα ura3Δ0 his3Δ1 leu2Δ0 LYS2 met15Δ0 bet1Δ*::KAN, pBET1 [*2μ URA3 BET1*]), SARY143 (*MATα bet1-1 his3 ura3-52 LYS6 LEU2 TRP1*), SARY160 (*MATα ura3-52 leu2-3, -112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 sft1Δ::LEU2*, pSFT1 [*2μ URA3 TPI-SFT1*]), SEY6210 (*MATα leu2-1,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9*) and DBY1 (*MATα sed5-1 leu2-1,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9*). Calcium chloride or electro-competent *Escherichia coli* DH10B cells were used to recover plasmids from yeast as well as for recombinant DNA experiments.

All yeast strains were grown at 25 or 37°C in either yeast extract–peptone–dextrose medium (YEPD), synthetic dextrose medium (SD) lacking the appropriate amino acids or on plates containing 5-fluoro-orotic acid (5-FOA, 100 μg/ml) as appropriate. Yeast transformations were performed using lithium acetate [6].

2.2. Yeast methods and functional complementation

Yeast media were prepared as described in [7]. SARY130 cells (*sft1-1*) were transformed with an *A. thaliana* cDNA library constructed in the vector pFL61 in which expression was driven by the phosphoglycerate kinase (*PGK*) promoter (ATCC # 77500) [8]. Transformed yeast cells were plated onto SD medium lacking uracil and incubated at 25°C until colonies were just visible (1.5–2 days) after which the plates were removed to a 37°C incubator. Plasmid DNA was extracted from colonies that appeared after 2–3 days of incubation at 37°C and recovered by transformation of *E. coli* by electroporation. The ability of the plasmids to suppress the temperature-sensitive growth defect of the *sft1-1* strain was verified by re-testing independent isolates.

2.3. Molecular biology

Restriction and modifying enzymes were purchased from New England Biolabs. *Pfu* polymerase was purchased from Stratagene and *Taq* polymerase from Life Sciences. Nucleic acids were purified with reagents purchased from Qiagen. DNA sequence determination was performed using cycle sequencing and an ABI 310 automated DNA sequencer. Molecular cloning was accomplished using established techniques [9]. AtBS14a and 14b open reading frames were amplified using the polymerase chain reaction (PCR) and the synthetic deoxyribonucleotide primers: BS14a5′ (5′-ACCAGAATTCATGAATCCTAGAAGGGAGCC-3′) and BS14a3′ (5′-ACCAGGATCCTTACCGAGTAAGATAGTATAT-3′) to amplify the AtBS14a open reading frame and BS14b5′ (5′-ACCAGAATTCATGAACTTTCGAAGGGAGAA-3′), BS14b3′ (5′-ACCAGGATCCTTACCTAATAAGGTAATACA-3′), and FL615′ (5′-ACCAGCGGCCGCTACAGATCATCAAGGAAGTA-3′) to amplify the AtBS14b open reading frame. pFL61 library DNA or pFL61 library isolates were used as template for PCR reactions. In any case, for expression in yeast, PCR-generated DNA fragments were digested with *Eco*RI and *Bam*HI and ligated into the similarly digested 2μ (multi-copy) yeast vectors pRS425T (2μ *LEU2*, pTPI), pWT1 (2μ *HIS3*, pTPI), pWT2 (2μ *URA3*, pTPI), or pRS424T (2μ *TRP1*, pTPI). Site-directed mutagenesis was performed essentially as described in [18] using the following

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[1] The cDNA sequences of AtBS14a and BS14b have been deposited in GenBank (accession numbers AF368175 and AY033334 respectively).

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deoxyribonucleotide primers: 14aXhoI (5′-GAGCGTGCCCTCGAGGGATTGCAA-3′) and 14bXhoI (5′-GATGAAGCTCTCGAGAATCTTCAG-3′) to introduce a *Xho*I site into the cDNAs of AtBS14a and BS14b respectively. Chimera were generated using standard methods following the scheme presented in Fig. 4A.

2.4. Electron microscopy

Yeast strains (SARY160, SEY6210 and SARY387 (*MATα sft1Δ::LEU2 leu2-1,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9* [*2μ, TRP1, pTPI-AtBS14a*]) were grown at 30°C in YEPD to an OD_{660} of 0.6. Cells were fixed, dehydrated and embedded in Spurr's resin as described previously [10]. Ultra-thin sections were prepared using a Leica Wild M3Z ultra-microtome, stained with lead citrate and uranyl acetate and viewed (at 100 kV) and photographed using a Philips CM20.

2.5. In vitro binding studies

Plasmids for the expression of yeast Golgi SNAREs in *E. coli* as well as the expression conditions and the in vitro mixing assay have been described previously [10,15]. The soluble forms of AtBS14a and AtBS14b lacking their hydrophobic COOH-termini (amino acid residues 103–122 and 107–123 respectively) were expressed as GST fusion proteins in pGEX2T (Pharmacia).

3. Results and discussion

3.1. AtBS14a is a dosage suppressor of the temperature-sensitive growth defect in sft1-1 cells

From the 180 000 library transformants screened six plasmids were identified that could suppress the temperature-sensitive growth defects of SARY130 cells, all of which contained a ~750 bp insert. DNA sequence determination of the insert from one of these plasmids revealed a 681 bp cDNA fragment

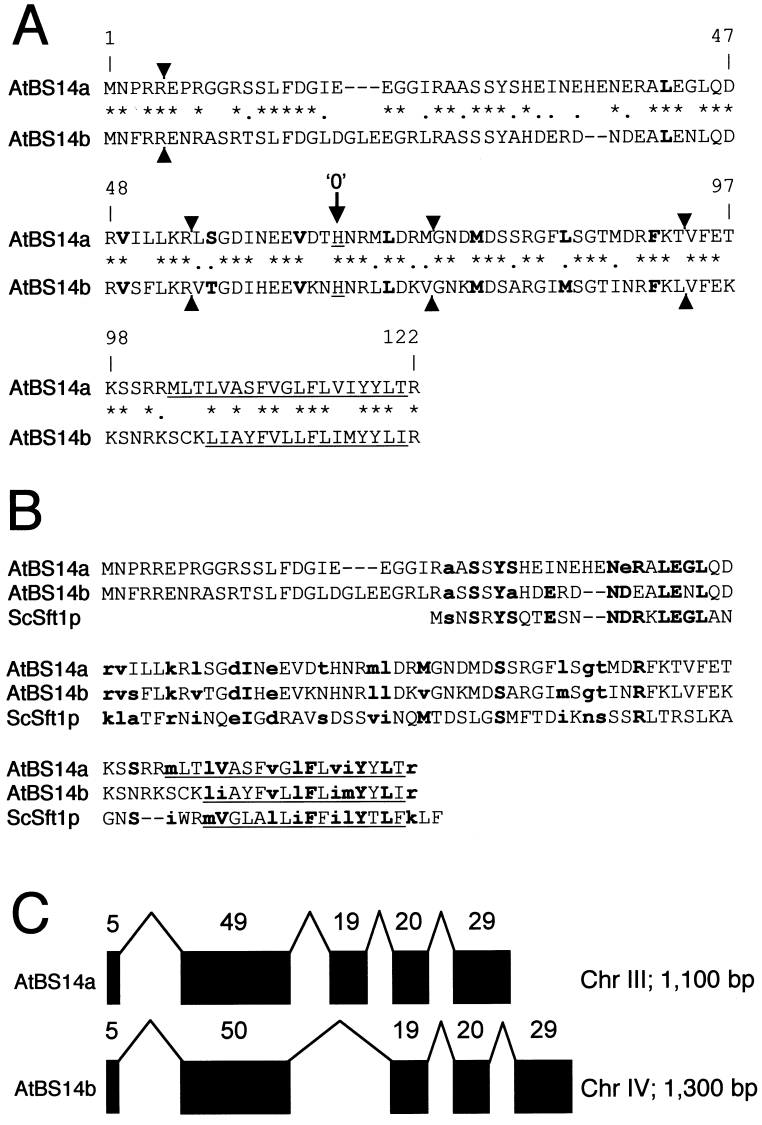

Fig. 1. The amino acid sequences and gene structures of AtBS14a and AtBS14b. (A) Alignment of the predicted amino acid sequences of AtBS14a and 14b. Arrowheads correspond to the position of introns and the arrow indicates the position of the zero ionic layer residues, which are underlined. The first and seventh position of the heptad repeats are indicated in bold. The hydrophobic C-termini are underlined. (B) Alignment of the predicted amino acid sequences of AtBS14a and 14b with Sft1p. Upper and lower case bold fonts denote amino acid identity and similarity (respectively) between Sft1p and AtBS14a or AtBS14b. (C) The gene structures of AtBS14a and AtBS14b. The AtBS14a (accession number AL137081.1) and AtBS14b genes (accession number Z97336.1) are located on *A. thaliana* chromosomes 3 and 4 respectively. Solid squares denote exons and the numbers of amino acids in each exon are shown above.

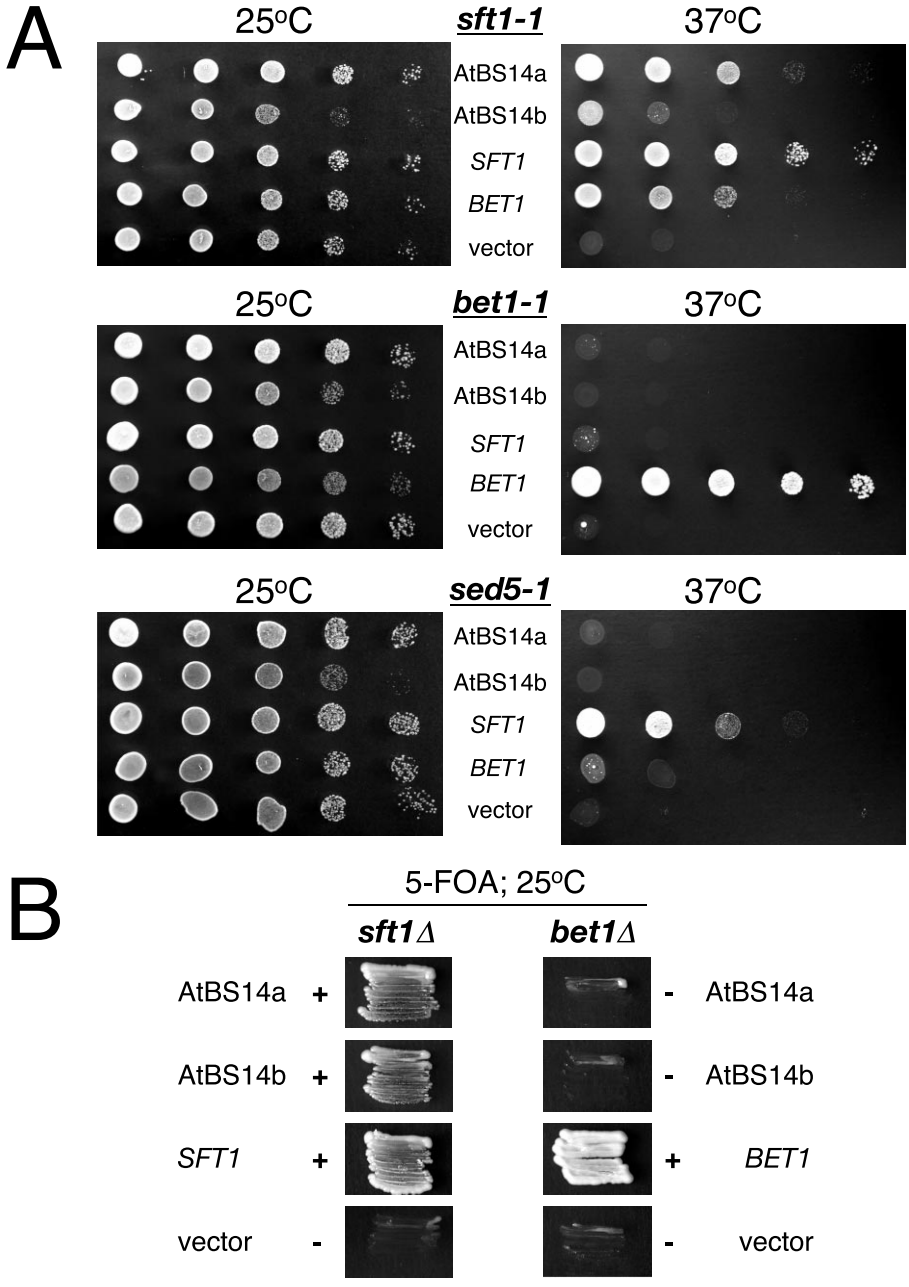

Fig. 2. Dosage suppression profiles of AtBS14a and AtBS14b for various yeast Golgi SNARE mutants. Yeast cells were transformed with 2μ plasmids containing AtBS14a, AtBS14b, *SFT1*, *BET1* or empty vector as indicated. (A) AtBS14a and AtBS14b are dosage suppressors of the temperature-sensitive growth defects in *sft1-1* cells. Decreasing numbers of yeast cells were spotted (from left to right) in replica onto minimal media plates and incubated at either 25°C or 37°C for 3 days. (B) AtBS14a and AtBS14b support the growth of *sft1*Δ but not *bet1*Δ cells. SARY160 (*sft1*Δ) or SARY270 (*bet1*Δ) cells were transformed with 2μ plasmids containing AtBS14a, AtBS14b, *SFT1* or empty vector, as indicated. Cells were patched onto plates containing 5-FOA and incubated at 25°C for 3 days. The + symbol indicates cell growth and the − symbol no growth. The doubling times of these strains at 30°C (in YEPD) were: *sft1*Δ pSFT1, 1.8 h; *sft1*Δ pAtBS14a, 2.0 h; *sft1*Δ pAtBS14b, 3.3 h; and wild-type (SEY6210), 1.7 h.

predicted to encode a 122 amino acid protein with typical SNARE-like features which we have designated AtBS14a, for *A. thaliana B*et1p/*S*ft1p-like protein of predicted molecular mass 14 kDa. AtBS14a is identical to hypothetical protein F9D24.80 (NCBI). We subsequently determined that all of the six plasmid isolates were AtBS14a cDNAs. By BLAST [11] AtBS14a is most similar to members of the mammalian Bet1 and GS15 protein families (sharing ~35% and ~50% amino acid sequence identity and similarity respectively). Strikingly AtBS14a shares modest overall amino acid homology with Sft1p (16% identity), and beyond obvious SNARE-like features shares no significant amino acid similarity to Bet1p.

3.2. *Arabidopsis contains an additional member of the BS14 family*

A search of public databases identified a genomic DNA fragment from *A. thaliana* which corresponded to the AtBS14a gene, as well as an additional genomic DNA clone very similar to AtBS14a which we have designated AtBS14b. An

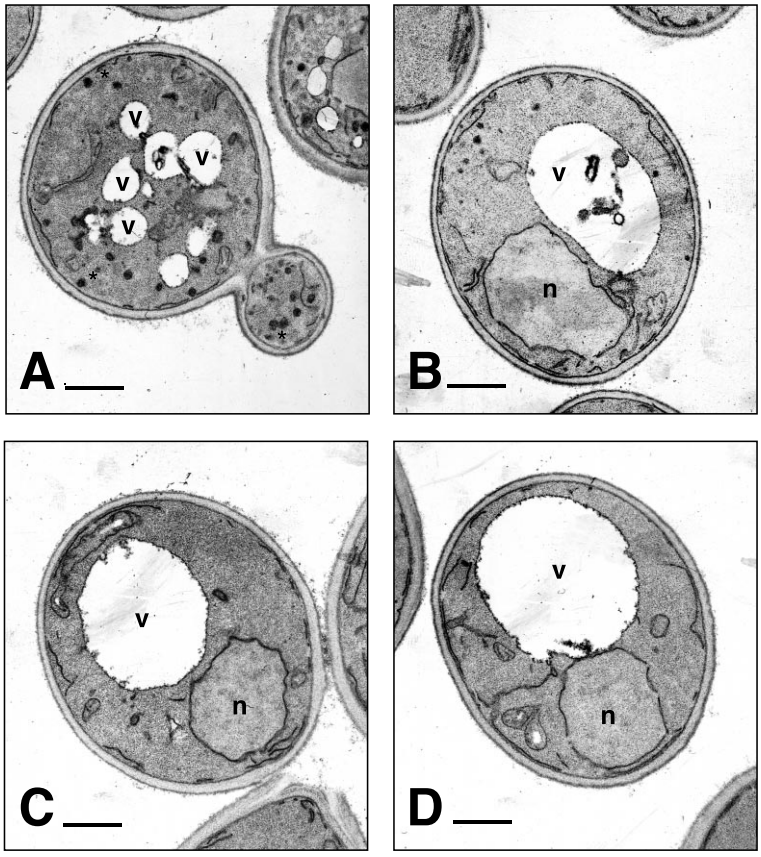

Fig. 3. Yeast cells expressing AtBS14a in place of Sft1p exhibit some defects in intra-cellular membrane organization. (A and B) *sft1*Δ cells containing the 2μ pTPI-AtBS14a plasmid. (C) *sft1*Δ cells containing the 2μ pTPI-*SFT1* plasmid. (D) Wild-type cells (SEY6210). The scale bar is 1 μm.

alignment of the predicted amino acid sequences of AtBS14a and AtBS14b is presented in Fig. 1A. AtBS14a and AtBS14b share 60% and 78% identity and similarity respectively at the amino acid sequence level. Interestingly, like Sft1p (aspartic acid) and Bet1p (serine) AtBS14a and AtBS14b also contain a non-canonical amino acid (histidine) at the so-called zero ionic layer of their core domains [12] rather than a glutamine, which has been used to define this class of SNAREs as Q-SNAREs [13] (Fig. 1A). Substitution of serine or aspartic acid for histidine at these positions (by site-directed mutagenesis) did not alter the ability of either AtBS14a or AtBS14b to support the growth of *sft1*Δ or *bet1*Δ cells at 25°C (W. Tai and D. Banfield, unpublished observations). An alignment of the predicted amino acid sequences of AtBS14a and AtBS14b with Sft1p is presented in Fig. 1B.

The structures of the AtBS14a and AtBS14b genes were deduced using the predicted amino acid sequences derived from the sequenced cDNAs together with the use of splice acceptor/donor site consensus sequences (Fig. 1C). Despite being located on separate chromosomes, the organization of the AtBS14a and 14b genes is very similar (see Fig. 1C). Indeed, although the size and nucleotide sequences of the introns differ in their overall length and composition, the positions of the splice sites as well as the sizes of the exons are almost identical (see Fig. 1A,C). These data suggest that AtBS14a and AtBS14b may represent recently duplicated genes, the products of which may (or may not) be functionally redundant in *Arabidopsis*. In this regard, no additional members of either the *BET1* or *SFT1* SNARE family were identified in a recent analysis of the *A. thaliana* genome, in which AtBS14a and AtBS14b have also been termed AtBet1 1 and AtBet1 2 [14].

We subsequently determined by PCR that AtBS14b cDNAs were present in the pFL61 library, confirming that the AtBS14b gene is expressed. However unlike AtBS14a, pTPI-AtBS14b only partially suppresses the temperature-sensitive growth defect in *sft1-1* cells. Thus AtBS14a and AtBS14b differ in their ability to suppress the temperature-sensitive growth defects of *sft1-1* cells (Fig. 2A).

SFT1 was originally identified as a dosage suppressor of the temperature-sensitive growth defect in *sed5-1* cells, a property that *BET1* does not share (Fig. 2A). In addition, we have previously shown that *BET1* is a dosage suppressor of the temperature-sensitive growth defects in *sft1-1* cells [15]. To determine whether AtBS14a and AtBS14b might be orthologues of either *SFT1* or *BET1* we asked whether AtBS14a or AtBS14b were multi-copy suppressors of the temperature-sensitive growth defect in *sed5-1* or *bet1-1* cells. AtBS14a and AtBS14b are not dosage suppressors of the temperature-sensitive growth defect of either *bet1-1* or *sed5-1* cells (Fig. 2A).

3.3. Either AtBS14a or AtBS14b can directly substitute for SFT1 but not BET1

A more direct test to address the functional equivalence of AtBS14a and AtBS14b is to determine whether either gene

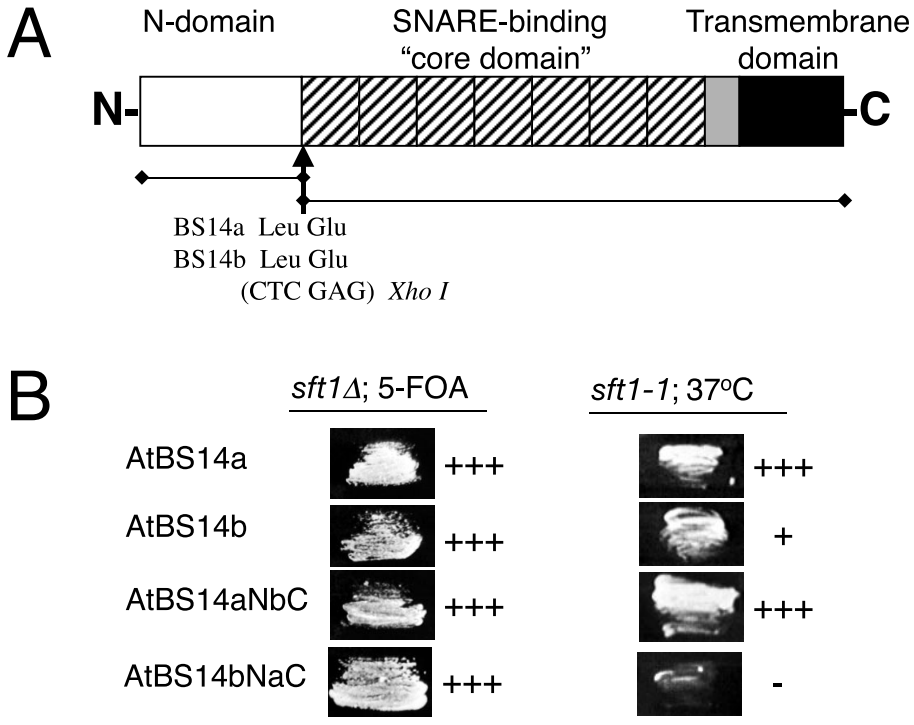

Fig. 4. Generation of AtBS14a and AtBs14b chimera. (A) Scheme used to generate AtBS14a and AtBS14b core and N-terminal chimera. (B) The molecular determinants underlying the observed differences in dosage suppression of the temperature-sensitive growth defect in *sft1-1* cells by AtBS14a and AtBS14b do not reside in the core domain. SARY160 (*sft1*Δ) and SARY130 (*sft1-1*) cells were transformed with 2μ plasmids containing AtBS14a, AtBS14b, or their respective chimera as indicated. The + symbols indicate relative growth rates and the − symbol indicates no growth.

can support the growth of a strain lacking the equivalent yeast gene.

Either AtBS14a or AtBS14b can act as dosage suppressor of the lethality associated with deletion of the *SFT1* gene but not of deletion of the *BET1* gene (Fig. 2B). This property of AtBS14a and AtBS14b is also evident on low copy number plasmids (*CEN6*) when gene expression is under the influence of the relatively strong triose phosphate isomerase (*TPI*) promoter (results not shown). However *CEN6* levels of either AtBS14a or AtBS14b, where expression is under control of the relatively weak *SFT1* promoter, were not sufficient to support the growth of *sft1*Δ cells (results not shown). These results are reminiscent of those obtained for gene dosage experiments with *BET1* in which 2μ *BET1* but not *CEN6 BET1* supported the growth of *sft1*Δ cells ([10] and M. Tsui and D. Banfield, unpublished observations). The abilities of AtBS14a and AtBS14b to complement mutations in *SFT1*, *BET1* or *SED5* are summarized in Fig. 2.

3.4. Yeast cells expressing AtBS14a in place of Sft1p exhibit some defects in intra-cellular membrane organization

To examine the extent to which over-expression of AtBS14a could compensate for the absence of Sft1p we examined *sft1*Δ pTPI-AtBS14a cells by electron microscopy (Fig. 3). The phenotypes of *sft1*Δ pTPI-AtBS14a cells were variable. Some cells had fragmented vacuoles and numerous darkly staining structures (Fig. 3A) reminiscent of the phenotype of *sft1-1* cells. Whereas others had intact vacuoles and fewer darkly staining

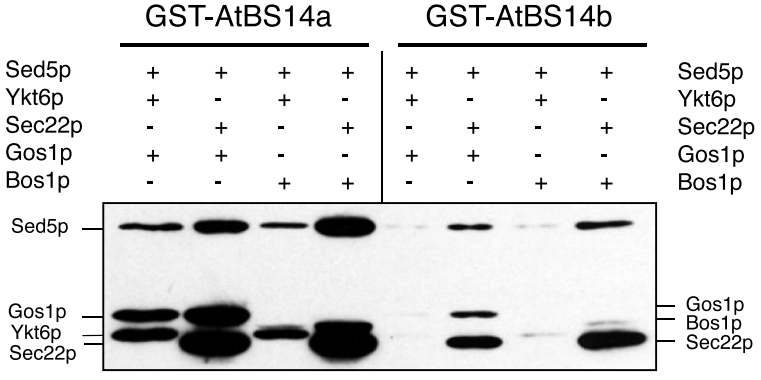

Fig. 5. AtBS14a and AtBS14b form complexes with yeast Golgi SNAREs in vitro. GST-AtBS14a or GST-AtBS14b were mixed with equal amounts of $(His)_6$-Sed5p and various combinations of two other $(His)_6$-tagged SNARE proteins as indicated. Proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes and recombinant $(His)_6$-tagged SNAREs detected by immuno-staining using an anti-$(His)_6$ antibody [10].

structures resembling cells that over-express Sft1p (Fig. 3B,C). The appearance of the cells in Fig. 3A,B may reflect differences in the amounts of AtBS14a protein in these cells. Like *sft1*Δ pTPI-*BET1* and *sft1*Δ pTPI-*SNC2* cells, *sft1*Δ pTPI-AtBS14a cells did not accumulate endoplasmic reticulum membranes [10].

3.5. Are AtBS14a and AtBS14b functionally redundant?

Evidence in *Saccharomyces cerevisiae* that over-expression of either *Bet1p* or *Snc2p* can bypass the requirement for *SFT1* [10] suggests that at least under some circumstances *Sft1p* can be functionally redundant. We have also used temperature-sensitive alleles of *SED5* (*sed5-1*) and *BET1* (*bet1-1*) to identify additional members of the Sft1/Bet1 family, as well as the corresponding orthologues from *A. thaliana*. No dosage suppressors of the temperature-sensitive growth defects of the *bet1-1* strain were identified. Similarly, a screen for dosage suppressors of the temperature-sensitive growth defect in *sed5-1* cells did not result in the isolation of either of the *A. thaliana Sed5*-like proteins (AtSYP51 and AtSYP52, [14]) but curiously did identify a SNAP25-like encoding cDNA (accession number CAB52583) that could partially suppress the temperature-sensitive phenotype. The apparent absence of any additional *Bet1*/*Sft1* family members in the *A. thaliana* genome suggests that like *S. cerevisiae*, functional redundancy or functional overlap may also exist for this SNARE family in *A. thaliana*. Indeed, similar results have been described previously for two other closely related *A. thaliana* SNAREs, AtVti1a and AtVti1b, with respect to their ability to rescue the temperature-sensitive growth defects and secretion anomalies of various *vti1* alleles [16]. We have tested the ability of AtBS14a or AtBS14b to act as dosage suppressors of other *sft1* (*sft1-13* and *sft1-15*) and *sed5* (*sed5-2*, *sed5-3*, *sed5-4* and *sed5-5*) temperature-sensitive alleles. As was the case for *sed5-1*, neither AtBS14a nor AtBS14b suppressed the temperature-sensitive growth defects in any of the additional *sed5* alleles tested. In contrast AtBS14a and AtBS14b were allele-specific suppressors of the temperature-sensitive growth defects of the *sft1* alleles (W. Tai and D. Banfield, unpublished observations).

To address the issue of possible functional redundancy we constructed chimera between AtSB14a and AtBS14b in which either the N-terminal or core domains of the proteins were exchanged (Fig. 4A). Interestingly, the differences in the ability of AtBS14a and AtBS14b to suppress the temperature-sensitive growth defects in *sft1-1* cells appear to be independent of the sequence of the core domain (Fig. 4B). Plausible explanations for the observed differences between AtBS14a and AtBS14b could include preferential degradation of AtBS14b (or AtBS14NbCa), differences in the intra-cellular localization of these proteins or differences in their abilities to form complexes with yeast SNAREs.

3.6. AtBS14a and AtBS14b form SNARE complexes in vitro with yeast Golgi SNAREs

To address whether the *Arabidopsis* SNAREs could interact directly with and form SNARE complexes with yeast Golgi SNAREs we carried out in vitro mixing experiments using bacterially expressed AtBS14a and AtSB14b together with the yeast Golgi SNAREs Sed5p, Gos1p, Bos1p, Sec22p, and Ykt6p. Both AtBS14a and AtBS14b formed quaternary SNARE complexes with yeast Golgi SNAREs (Fig. 5). Interestingly, AtBS14a resembled Sft1p in its yeast SNARE binding profiles while AtBS14b resembled those of Bet1p [10]. We conclude that the inability of AtBS14a or AtBS14b to either suppress the temperature-sensitive growth defects in the *bet1-1* strain or support the growth of *bet1*Δ cells is unlikely to be exclusively related to the ability of these proteins to form complexes with the yeast SNAREs in vivo [16,17].

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