FEBS Letters 566 (2004) 218–222 FEBS 28400

Complementation of *Saccharomyces cerevisiae ccc2* mutant by a putative P_{1B}-ATPase from *Brassica napus* supports a copper-transporting function

Jennafer L. Southron*, Urmila Basu, Gregory J. Taylor

Department of Biological Sciences, University of Alberta, Edmonton, Alta., Canada T6G 2E9

Received 12 March 2004; revised 19 April 2004; accepted 19 April 2004

Available online 28 April 2004

Edited by Ulf-Ingo Flügge

Abstract Copper transport across membranes plays an important role in plant growth and survival. P_{1B}-type ATPases participate in transmembrane transport of copper in various organisms. A *Brassica napus* cDNA (*BnRAN1*) encoding a putative Cu^{2+}-ATPase was cloned in this study. A complementation assay demonstrated that the protein encoded by this cDNA could functionally replace Ccc2p, a *Saccharomyces cerevisiae* Cu^{2+}-ATPase, rescuing growth of *ccc2* mutant under iron-limited conditions. Our results suggest that this rescue likely resulted from restoration of copper delivery, mediated by BnRAN1, to Fet3p. This study is amongst the first to demonstrate that a putative plant P_{1B}-ATPase is functional and to examine its substrate specificity.

Keywords: Copper; P-type ATPase; CPx-ATPase; P_{1B}-ATPase; CCC2; RAN1

1. Introduction

Copper is an essential micronutrient and copper-containing proteins play fundamental roles in the survival of plants [1]. Copper is a cofactor involved in respiration (cytochrome-*c* oxidase, alternate oxidase), photosynthesis (plastocyanin), and the detoxification of superoxide radicals (copper/zinc superoxide dismutase). While copper is required in limited amounts, it is toxic in excess [1] and mechanisms are required to control the amount and location of copper within cells. Transport of copper across membranes plays an important role in maintaining this copper homeostasis. A P_{1B}-ATPase, Ccc2p, plays a role in copper and iron homeostasis in *Saccharomyces cerevisiae*. The Ccc2 protein delivers copper to the multicopper oxidase Fet3p, which is required for high-affinity iron uptake [2] (Fig. 1). Similar roles for P_{1B}-ATPases have been reported in other eukaryotes, including *Caenorhabditis elegans* [3] and humans [4].

Members of the P-type ATPase superfamily transport charged substrates across membranes. This transport is ATP-dependent and involves the phosphorylation of an aspartate (D) residue in the conserved amino acid sequence DKTGT[LIVM][TI] (Prosite PS00154). The P-type ATPase superfamily clusters on a phylogenetic tree according to substrate specificity [5] and has been divided into five major branches (Type 1–5), which may be further divided into subfamilies. The Type 1B subfamily (P_{1B}-ATPases) is made up of at least two groups, the Cd^{2+}-(Zn^{2+}-/Co^{2+}-/Pb^{2+}-) and Cu^{2+}-(Ag^{2+}-) ATPases [5]. Putative P_{1B}-ATPase can be identified based on sequence similarity to known P_{1B}-ATPases as well as the presence of Type 1B specific amino acid motifs, including metal-binding region(s), a CPx motif, and a HP dipeptide [6]. Amino acid residues that may play a role in determining the substrate specificity of Cu^{2+}-ATPases have also been identified. A leucine (L) residue located 21 amino acids C-terminal of the CxxC metal-binding motif(s) [7] and an extended CPx motif (CPC[AS]LGLATP) [8] have been proposed for copper-transporting P_{1B}-ATPases.

While a great deal of research has focused on Cu^{2+}-ATPases from organisms such as humans (ATP7A and ATP7B) and *S. cerevisiae* (Ccc2p), information about Cu^{2+}-ATPases from plants is relatively limited. When this study was initiated an *Arabidopsis* cDNA encoding a putative P_{1B}-ATPase named PAA1 had been cloned, but information about its substrate specificity and function were minimal [9]. Three additional *Arabidopsis* sequences encoding putative P_{1B}-ATPases could be identified using the BLAST [10] family of programs. One of these putative P_{1B}-ATPases (AC002342 genomic locus T19K24.18; At5g44790) could be predicted to transport copper. More recent studies focusing on two groups of allelic mutants (*ran1* and *paa1* mutants) and their corresponding wild type genes (*RAN1* and *PAA1*; identified by map-based cloning) suggest that the putative *Arabidopsis* P_{1B}-ATPases RAN1 (At5g44790) and PAA1 are involved in copper delivery to ethylene receptors [11,12] and across the chloroplast envelope [13], respectively. To further investigate whether P_{1B}-ATPases play a role in copper transport in plants, we cloned a cDNA that encodes a homolog of the putative *Arabidopsis* Cu^{2+}-ATPase At5g44790 from *Brassica napus*. A complementation assay was used to demonstrate that the protein encoded by the *B. napus* cDNA could functionally replace Ccc2p, a Cu^{2+}-ATPase from *S. cerevisiae*, providing support for a copper-transporting function.

2. Materials and methods

2.1. Cloning a B. napus cDNA that encodes a putative P_{1B}-ATPase

A partial cDNA (2323 bp) was isolated from a *B. napus* cDNA library kindly provided by Dr. Isobel Parkin (Agriculture and

* Corresponding author. Fax: +1-780-492-9234.
E-mail address: jsouthro@ualberta.ca (J.L. Southron).

0014-5793/$22.00 © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2004.04.035

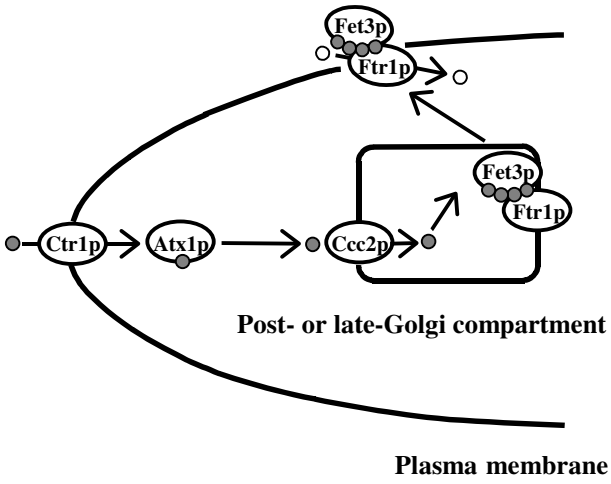

Fig. 1. Model illustrating the role of Ccc2p in copper and iron homeostasis of *S. cerevisiae*. Ccc2p (Cu^{2+}-ATPase) transports copper (●) into a late- or post-Golgi compartment where copper is incorporated into Fet3p. Maturation of Fet3p through the secretory pathway requires Ftr1p. Mature Fet3p (multicopper oxidase) and Ftr1p (iron permease) function at the plasma membrane in high-affinity iron (○) uptake. Ctr1p (high-affinity copper uptake) and Atx1p (copper chaperone) participate in copper delivery to Ccc2p. Figure adapted from [16]. For additional details see [27].

Agri-Food Canada). This library was generated using the Uni-ZAP® XR Cloning Kit (Stratagene) and mRNA isolated from five-day-old, etiolated, *B. napus* seedlings from the double haploid line DH12075. An oligonucleotide probe, produced using the primers 5′-AGTGAACACCCATTGGCAA-3′ and 5′-CTTTGGCGACTGCTCTTGC-3′, was used to screen the library following amplification.

The 5′-end missing from the partial cDNA was isolated using RT-PCR. The SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase Kit (Gibco), a gene specific primer (5′-ATGTATCACCAGGCTGAA-3′), and total RNA isolated from leaves of the *B. napus* cultivar Westar were used to produce first strand cDNA. The PCR step used HotStarTaq™ DNA Polymerase (Qiagen), and sense (5′-AAGAAATGGCGCCGAGTAGAC-3′) and antisense (5′-ATCGCTGTTGCTGGAGTAAGT-3′) primers.

The RT-PCR product (1356 bp) was ligated to the partial cDNA (2323 bp) using a *Sac*I site located in a region of overlap shared by both fragments. The resulting full-length cDNA was cloned into *Xho*I/*Xba*I digested pBluescript SK- vector and sequenced. Sub-cloning (*Hin*dIII/*Bam*HI) into pYES3 vector (Invitrogen) produced the pYES3-cDNA vector. Transcription of sequences cloned into pYES3 is mediated by the *GAL1* promoter and is induced in the presence of galactose, repressed by glucose, and unaffected by raffinose.

2.2. Saccharomyces cerevisiae *strains and growth conditions*

Established techniques [14] were used in the growth, maintenance, and manipulation of the *S. cerevisiae* strains. The *S. cerevisiae* strains were obtained from Dr. Diane Cox (University of Alberta) [15,16]. All amino acids were added to synthetic medium for maintenance of the parental strain BJ2168 (*MAT***a** *pep4-3 prc1-407 prb1-1122 ura3-52 trp1 leu2*). The *CCC2*, *FET3*, and *CTR1* genes of BJ2168 were independently disrupted to create the *ccc2*, *fet3*, and *ctr1* stains, respectively [15,16]. These disruption strains were selected and maintained on synthetic medium containing all amino acids except uracil.

The *S. cerevisiae* strains BJ2168, *ccc2*, *fet3*, and *ctr1* were independently transformed with pYES3 or pYES3-cDNA vectors using a modified lithium acetate method [17]. Transformants were selected on synthetic plates supplemented with all amino acids except tryptophan.

2.3. Experimental (iron-limited, iron-sufficient, and copper-sufficient) media

The iron-limited, iron-sufficient, and copper-sufficient media were modified synthetic media [14] based on media described by Forbes and Cox [16]. The carbon source was changed, from 2% glucose to 2% galactose and 1% raffinose, to allow transcription from the pYES3 vector. Iron-limited medium was made with YNB that lacked both $CuSO_4$ and $FeCl_3$ (Bio-101), but was supplemented with 50 mM MES buffer (pH 6.1), 1 mM ferrozine, 50 μM $Fe(NH_4)_2(SO_4)_2$, and 1 μM $CuSO_4$. Copper- and iron-sufficient media were made by altering the iron-limited media so that it contained a final concentration of 500 μM $CuSO_4$ and 350 μM $Fe(NH_4)_2(SO_4)_2$, respectively. Ferrozine was omitted from the iron-sufficient medium.

2.4. Complementation assay and carbon source growth curve

For all growth assays, cultures grown to saturation in conventional synthetic medium were washed three times with sterile, deionized water (18 mΩ). For carbon source growth curves, washed cells were resuspended in sterile, deionized water and used to inoculate 5 ml aliquots of either conventional or induction (2% galactose and 1% raffinose in place of 2% glucose) synthetic medium at an OD_{600} of 0.1. Two types of complementation assays (plate assay, growth curves) were conducted. For complementation assays, washed cells were resuspended in induction medium, incubated 16 h, washed, resuspended in iron-limited medium, incubated 16 h, washed, and resuspended in sterile, deionized water (plate assay) or iron-limited medium (growth curves). For plate assays, cells were resuspended at an OD_{600} of 0.1 and 5 μl samples were spotted or spread onto experimental plates. For growth curves, cells were used to inoculate 5 ml of experimental media at an OD_{600} of 0.1. OD_{600} values for growth curves were determined in a 96-well microplate using 200 μl samples. Each experiment was performed twice and representative results are shown.

3. Results and discussion

3.1. A Brassica napus cDNA that encodes a putative P_{1B}-ATPase

A full-length, *B. napus* cDNA (3194 bp) was produced by ligating a RT-PCR product (1356 bp) and a partial clone from a cDNA library (2323 bp). Sequence data from a 485 bp region of overlap supported a common origin for these fragments. The full-length cDNA sequence was submitted to GenBank (AY045772). The amino acid sequence (AAL02122) predicted from the *B. napus* cDNA contained the phosphorylation site (DKTGTLT) characteristic of the P-type ATPase superfamily. Motifs suggested to be characteristic of P_{1B}-ATPases [6], including two metal-binding regions (CAAC), the CPx motif (CPC), and the HP dipeptide were also present. The predicted amino acid sequence was most similar to the Cu^{2+}-ATPases, including ATP7A and ATP7B [4], containing leucine (L) residues 21 amino acids C-terminal of the two CxxC (CAAC) motifs as well as the extended CPx motif (CPCALGLATP). Thus, copper was considered the likely substrate for this putative P_{1B}-ATPase.

3.2. Complementation of the S. cerevisiae *ccc2 mutant*

Complementation assays utilizing *S. cerevisiae* strains with the disrupted *CCC2* gene have been developed to study putative Cu^{2+}-ATPases [3,15,16,18]. The Ccc2 protein is localized to a late- or post-Golgi compartment in the secretory pathway [19], where it delivers copper to the multicopper oxidase Fet3p [2,19]. Copper loaded Fet3p is required at the plasma membrane for high-affinity iron uptake [20]. *S. cerevisiae* strains with the disrupted *CCC2* gene produce copper-deficient Fet3p and are, consequently, deficient in high-affinity iron uptake [2]. A functional high-affinity iron uptake system is required for growth under iron-limited conditions. The ability of a putative Cu^{2+}-ATPase to rescue a *ccc2* mutant under iron-limited conditions has been interpreted as evidence, suggesting that the protein transports copper [3,15,16,18]. Under iron-replete

conditions, iron uptake occurs via a low-affinity uptake system [21] that is not dependent upon Ccc2p.

The growth pattern observed on iron-limited plates (Fig. 2A) and in iron-limited medium (Fig. 3A) suggested that the putative Cu^{2+}-ATPase encoded by the *B. napus* cDNA is capable of complementing the *ccc2* mutant. The *ccc2* strain transformed with the pYES3-cDNA vector was able to grow, while the *ccc2* strain transformed with pYES3 did not grow (Figs. 2A and 3A). The *ccc2*/pYES3-cDNA strain did not grow when galactose and raffinose were replaced by glucose (data not shown).

Confirmation of a role in copper transport, however, requires invalidation of another hypothesis. When the *CCC2* gene is disrupted, Fet3p does not receive the copper it requires to function and the *ccc2* mutant is unable to grow on iron-limited plates because of the resulting impairment of high-affinity iron uptake. Thus, it is possible that the *B. napus* cDNA rescued the *ccc2* mutant by functionally replacing inactive Fet3p, or by providing alternative iron uptake activity. To test these alternative hypotheses, the *fet3* mutant was

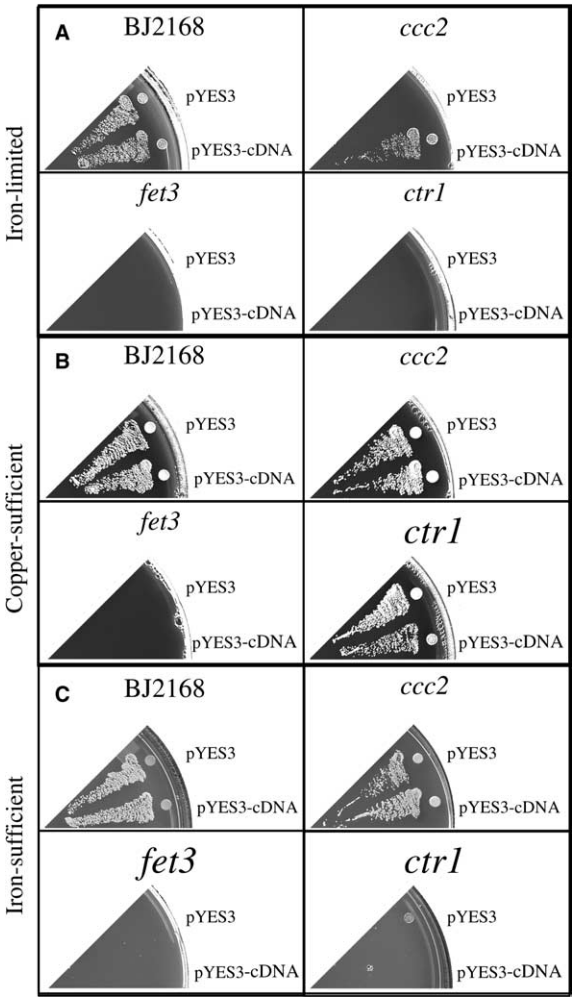

Fig. 2. Growth of BJ2168, *ccc2*, *fet3*, and *ctr1* mutants transformed with vector (pYES3) or vector carrying the *B. napus* cDNA (pYES3-cDNA) on iron-limited (A), copper-sufficient (B), and iron-sufficient (C) plates. Plates were incubated at 30 °C for 72 h. Sections shown in A, B, and C are from single plates, representative ($n = 3$) of each type of media.

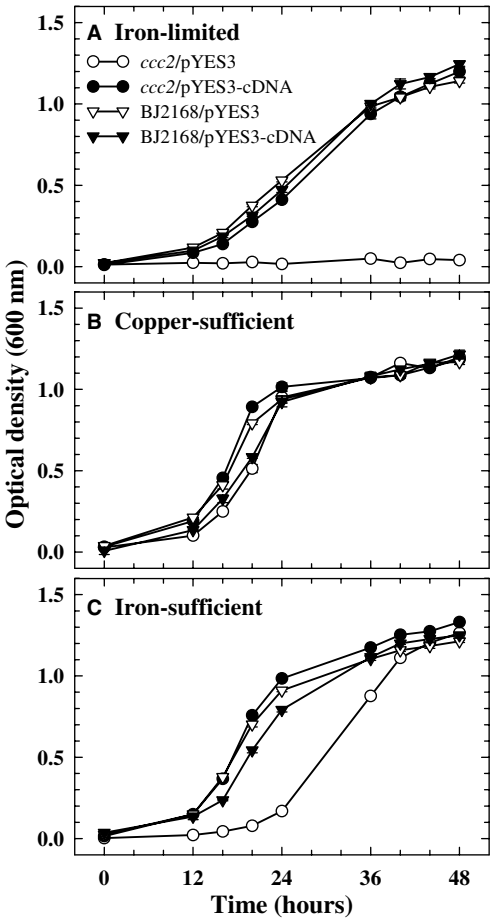

Fig. 3. Growth curves for BJ2168 and *ccc2* mutant transformed with vector (pYES3) or vector carrying the *B. napus* cDNA (pYES3-cDNA) in iron-limited (A), copper-sufficient (B), and iron-sufficient (C) media. Data points represent an average OD_{600} ($n = 3$; ±S.E.).

transformed with either pYES3 or pYES3-cDNA. These *fet3* transformants did not grow on the iron-limited plates (Fig. 2A), ruling out the possibility that the *B. napus* cDNA resulted in a direct rescue of impaired high-affinity iron uptake.

Delivery of copper to Ccc2p is dependent upon copper uptake across the plasma membrane. Under copper-limited conditions, copper enters the cell by a high-affinity uptake system encoded by the *CTR1* [22] and/or *CTR3* [23] gene(s). Expression of the *CTR3* gene is eliminated in most *S. cerevisiae* laboratory strains and high-affinity copper uptake is dependent solely on Ctr1p [23]. Copper uptake under copper-replete conditions occurs via a low-affinity uptake system [22].

Similar to the *ccc2* mutant, the *ctr1* mutant is also deficient in copper transport. While the *ccc2* mutant is deficient in copper transport localized to the secretory pathway, the *ctr1* mutant is deficient in high-affinity copper uptake at the plasma membrane. High-affinity iron uptake is also impaired in the *ctr1* mutant, since copper is not delivered to Ccc2p and Fet3p. To determine if the protein encoded by the *B. napus* cDNA could mediate copper uptake at the plasma membrane, the *ctr1* mutant was transformed with either pYES3 or pYES3-cDNA. The transformants were unable to grow on the iron-limited plates (Fig. 2A), suggesting that the protein encoded by the *B. napus* cDNA was not able to mediate copper uptake across the plasma membrane.

As evident from growth curves of the BJ2168/pYES3 and BJ2168/pYES3-cDNA strains (Figs. 3A, B, and C), the protein encoded by the *B. napus* cDNA was neither detrimental nor beneficial (under the conditions tested) when Ccc2p was already present.

Previous studies have demonstrated that *ccc2*, *fet3*, and *ctr1* mutants can be rescued by supplementing an iron-limited medium with copper and/or iron [2,16]. This copper-/iron-mediated rescue can be used to demonstrate the viability of the strains that did not grow under iron-limited conditions. When the iron-limited medium was supplemented to 500 μM $CuSO_4$, all of the *ccc2* (Figs. 2B and 3B) and *ctr1* (Fig. 2B) strains grew. All of the *ccc2* strains also grew when the iron-limited medium was supplemented to 350 μM $Fe(NH_4)_2(SO_4)_2$ (Figs. 2C and 3C). There was no apparent growth of the *fet3* strains on iron-sufficient plates (Fig. 2C). A slower growth rate exhibited by *fet3* mutants, when glucose is replaced by galactose and raffinose (Fig. 4), was considered a possible explanation for this lack of apparent growth. When incubated for a longer period of time, the *fet3* mutants did grow on the iron-sufficient plates (Fig. 5). This extended growth period did not alter the results of the iron-limited plates (data not shown).

Although the *ccc2*, *fet3*, and *ctr1* mutants are all deficient in high-affinity iron uptake, the protein encoded by the *B. napus* cDNA was only capable of complementing the *ccc2* mutant. This supports a copper-transporting role that is likely localized to a secretory compartment for the product encoded by the *B. napus* cDNA.

3.3. Potential roles for the copper-transporting function encoded by the B. napus cDNA

Potential roles for P_{1B}-ATPase-mediated copper transport in plants have been suggested from work focusing on *Arabidopsis ran1* (*responsive-to-antagonist1*) mutants. The *ran1-1* and *ran1-2* mutants display an alteration in the specificity of the ethylene receptor(s) and contain mutations in the gene corresponding to the genomic locus T19K24.18 (AC002342; At5g44790) [11]. Expression of *RAN1* cDNA in *S. cerevisiae ccc2* mutants rescues these mutants [11]. These results led, in part, to a proposal that RAN1 functions in the delivery of copper to ethylene receptor(s) [11]. A rosette lethal phenotype, observed in the *Arabidopsis* mutant *ran1-3*, has been proposed to arise from an ethylene-independent pathway regulating cell expansion. This pathway is thought to be dependent upon cuproenzyme(s) that receive copper in a RAN1-dependent manner and that are, consequently, inactive in the *ran1-3* mutant [12]. It has also been suggested that RAN1 may play a role in copper recycling during senescence [24].

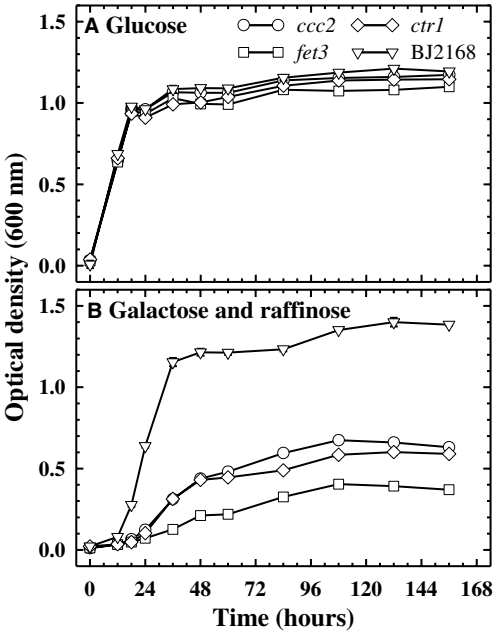

Fig. 4. Growth of BJ2168, *ccc2*, *fet3*, and *ctr1* mutants when the carbon source is glucose (A; conventional synthetic media) or galactose and raffinose (B; induction synthetic media). Data points represent an average OD_{600} ($n = 3$; ±S.E.).

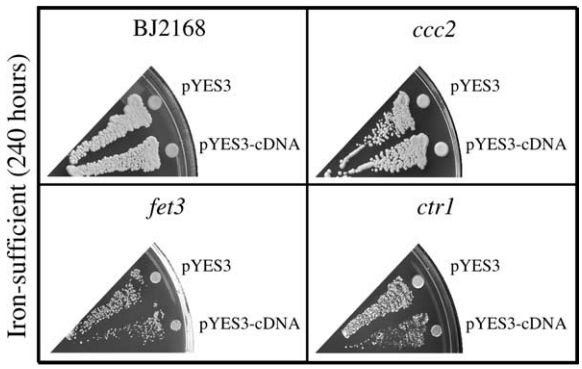

Fig. 5. Growth of BJ2168, *ccc2*, *fet3*, and *ctr1* mutants after the iron-sufficient plate shown in Fig. 2C was incubated for an additional 168 h (240 h total).

The *B. napus* and *RAN1* coding sequences are 88% identical, while the predicted amino acid sequences are 91% identical. This level of identity suggests that the *B. napus* cDNA may encode a RAN1 homolog. It is, consequently, likely that the protein encoded by the *B. napus* cDNA mediates functions similar to those described for RAN1. Results of the complementation assay in the current study suggest that the *B. napus* cDNA encodes a copper-transporting function. For these reasons, it is proposed that the *B. napus* gene (and cDNA) be named *BnRAN1*.

Four additional *Arabidopsis* sequences that encode putative P_{1B}-ATPases were added to the NCBI databases during the progress of this study. Partial sequences and expressed sequence tags, that appear to correspond to putative P_{1B}-ATPases, suggest that these ATPases occur in a variety of plant species. A recent paper [25] describes the identification of eight sequences encoding putative P_{1B}-ATPases from two draft sequences of the *Oryza sativa* (rice) genome. With the exception of the *Arabidopsis* P_{1B}-ATPases RAN1 [11,12] and recently PAA1 [13], AtHMA4 [26], and AtHMA3 [28], the functional information available for putative P_{1B}-ATPases in plants is still limited. The current study is, therefore, amongst the first studies to demonstrate that a putative plant P_{1B}-ATPase is functional and to examine its substrate specificity.

Acknowledgements: We thank Dr. Isobel Parkin for providing the *B. napus* cDNA library and Dr. Diane Cox for the *S. cerevisiae* strains. We also gratefully acknowledge the advice and assistance provided by Dr. Valar Anoop and Ms. Julie Stephens. Financial support was provided by the National Science and Engineering Research Council's research grants program.

References

[1] Maksymiec, W. (1997) Photosynthetica 34, 321–342.
[2] Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. and Klausner, R.D. (1995) Proc. Natl. Acad. Sci. USA 92, 2632–2636.
[3] Sambongi, Y., Wakabayashi, T., Yoshimizu, T., Omote, H., Oka, T. and Futai, M. (1997) J. Biochem. (Tokyo) 121, 1169–1175.
[4] Cox, D.W. (1999) Br. Med. Bull. 55, 544–555.
[5] Axelsen, K.B. and Palmgren, M.G. (1998) J. Mol. Evol. 46, 84–101.
[6] Solioz, M. and Vulpe, C. (1996) Trends Biochem. Sci. 21, 237–241.
[7] Gitschier, J., Moffat, B., Reilly, D., Wood, W.I. and Fairbrother, W.J. (1998) Nat. Struct. Biol. 5, 47–54.
[8] Tottey, S., Rich, P.R., Rondet, S.A. and Robinson, N.J. (2001) J. Biol. Chem. 276, 19999–20004.
[9] Tabata, K., Kashiwagi, S., Mori, H., Ueguchi, C. and Mizuno, T. (1997) Biochim. Biophys. Acta 1326, 1–6.
[10] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[11] Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A. and Ecker, J.R. (1999) Cell 97, 383–393.
[12] Woeste, K.E. and Kieber, J.J. (2000) Plant Cell 12, 443–455.
[13] Shikanai, T., Muller-Moule, P., Munekage, Y., Niyogi, K.K. and Pilon, M. (2003) Plant Cell 15, 1333–1346.
[14] Sherman, F. (1991) Methods. Enzymol. 194, 3–21.
[15] Forbes, J.R. (2000). Functional analysis of the copper-transporting P-type ATPase, ATP7B, defective in Wilson disease. PhD thesis. University of Alberta, Canada.
[16] Forbes, J.R. and Cox, D.W. (1998) Am. J. Hum. Genet. 63, 1663–1674.
[17] Elble, R. (1992) Biotechniques 13, 18–20.
[18] Payne, A.S. and Gitlin, J.D. (1998) J. Biol. Chem. 273, 3765–3770.
[19] Yuan, D.S., Dancis, A. and Klausner, R.D. (1997) J. Biol. Chem. 272, 25787–25793.
[20] Askwith, C., Eide, D., Van Ho, A., Bernard, P.S., Li, L., Davis-Kaplan, S., Sipe, D.M. and Kaplan, J. (1994) Cell 76, 403–410.
[21] Dix, D.R., Bridgham, J.T., Broderius, M.A., Byersdorfer, C.A. and Eide, D.J. (1994) J. Biol. Chem. 269, 26092–26099.
[22] Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. and Klausner, R.D. (1994) Cell 76, 393–402.
[23] Knight, S.A., Labbe, S., Kwon, L.F., Kosman, D.J. and Thiele, D.J. (1996) Genes Dev. 10, 1917–1929.
[24] Himelblau, E. and Amasino, R.M. (2000) Curr. Opin. Plant Biol. 3, 205–210.
[25] Baxter, I., Tchieu, J., Sussman, M.R., Boutry, M., Palmgren, M.G., Gribskov, M., Harper, J.F. and Axelsen, K.B. (2003) Plant Physiol. 132, 618–628.
[26] Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L. and Williams, L.E. (2003) Plant J. 35, 164–176.
[27] Eide, D.J. (1998) Annu. Rev. Nutr. 18, 441–469.
[28] Gravot, A., Lieutaud, A., Verret, F., Auroy, P., Vavasseur, A. and Richaud, P. (2004) FEBS Lett. 561, 22–28.