The Arabidopsis thaliana trehalase is a plasma membrane-bound enzyme with extracellular activity

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Abstract The lack of trehalose accumulation in most plant spe-cies has been partly attributed to the presence of an active treha-lase. Although trehalose synthesis enzymes are thought to be cytosolic, and previous studies have indicated that trehalase activity is extracellular, the exact location of the enzyme has not yet been established in plant cell. We present evidence that the yet uncharacterised full-length Arabidopsis trehalase is a plasma membrane-bound protein, probably anchored to the membrane through a predicted N-terminal membrane spanning domain. The full-length AtTRE1, when expressed in yeast can functionally substitute for the extracellularly active trehalase Ath1p, by sustaining the growth of an ath1 null mutant strain on trehalose and at pH 4.8. We further demonstrate that At-TRE1 expressed in yeast is plasma membrane-bound as in plant cell. In light of these findings, the regulation of plant cell endog-enous trehalose by trehalase is discussed.

Keywords: Arabidopsis; Plasma membrane; Trehalase; Trehalose; Yeast

1. Introduction

The disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing sugar found in organisms as diverse as bacteria, fungi, plants, and invertebrates but absent from mammals [1,2]. The function of this sugar may differ depending upon the organism, the growth or developmental stage considered. For instance, trehalose is the main reserve sugar of the hemolymph in flying insects but is also indispensable for thermotolerance of the larvae [2]. Trehalose synthesis and metabolism is also important for various microorganisms for pathogenesis and symbiotic interactions with their hosts [3,4]. But the most common role attributed to this uncommon sugar is its contribution to cell survival under stress conditions. Trehalose acts as a universal stabiliser of protein conformation due to its exceptional effect on the structure and properties of solvent water [5]. Hence, exogenous or engineered trehalose synthesis can protect human cells from redox or desiccation stress [6].

Trehalose or its derived metabolites are vital for embryo maturation in plant [7,8]. However, exogenous trehalose can inhibit plant growth by interfering with sugar sensing and metabolism [9–11]. Transgenic approaches suggest that it is possible to increase up to 10-fold the level of detectable trehalose in plant, this increase being correlated with enhanced tolerance to various abiotic stress [12,13]. As compared to naturally trehalose-accumulating plant species, the levels of trehalose obtained with transgenic plants remain relatively modest and this can be explained by specific trehalase activity which degrades trehalose, suggesting that one possible way to increase trehalose content in plant tissues might be to down-regulate trehalase activity [14]. Indeed, conclusive evidence of trehalose synthesis by non-desiccation tolerant plants was obtained only after treatment with the nitrogen-containing trehalose analogue but competitive inhibitor of trehalase, validamycin A [9,10,12].

The toxicity of exogenous trehalose has been mainly ascribed to signalling properties of the trehalose synthesis intermediate trehalose-6-phosphate (T-6-P) [15,16]. The available data suggest that besides T-6-P, intracellular (and extracellular) trehalose *per se* may be tightly regulated in plant cells. Catabolism of trehalose is mediated by trehalase. Although previous studies have indicated that trehalase activity is extracellular, the exact subcellular localisation of the enzyme has not yet been established in plant cell. Analysis of the available primary sequence of plant trehalases suggests that they do contain putative N-glycosylation sites [4,9], indicating that they may be secreted. Indeed, in suspension-cultured cells of soybean, it was shown that 80% of the total trehalase activity could be found in the cell-free medium [9]. From these observations and other circumstantial evidence it was thought that plant trehalases, including the Arabidopsis *At*TRE1, may be secreted and cell wall-associated [9,10]. However, the full-length cDNA of the Arabidopsis only known trehalase encodes a 626 aminoacids polypeptide as compared to the 565 aminoacids protein studied so far [10]. Furthermore, a transmembrane span is predicted within the first 61 residues of the full-length protein. The subcellular localisation of the Arabidopsis trehalase, and more importantly the compartmentation of its enzymatic activity are required to understand the molecular mechanisms of trehalose regulation in planta. In this

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Abbreviations: BY-2, tobacco bright yellow-cultured cells; GFP, green fluorescent protein; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulphate; YFP, yellow fluorescent protein

study, we used complementary approaches to demonstrate that the full-length *At*TRE1 is a plasma membrane-bound protein in plant cell and can functionally replace the extracellularly active yeast Ath1p.

2. Materials and methods

2.1. Genetic constructions

The validated full-length cDNA of At4g24040 [17] was obtained from RIKEN (Genomic Sciences Centre, Yokohama, Japan; clone RAFL19-66-E19). To generate a construct expressing the coding sequence of *At*TRE1 fused at its C-terminus to green fluorescent protein (GFP), the 1.9 kb *At*TRE1 cDNA was amplified with the primers TRE-HB019 5′-TAATTCTAGAACAATGAAATCATACAAACTTAATAAC and TRE-HB020 5′-ATTATCTCGAGACCGGCTTCAATGCTAAGATGAG, introducing XbaI and XhoI (underlined), respectively, at the 5′ and 3′ ends. The resulting PCR product was cloned into pVKH18-En6-NST-GFP [18] opened with XbaI/SalI, to generate *At*TRE1-GFP. To generate YFP-*At*TRE1, the *At*TRE*1* cDNA was amplified with TRE-HB021 5′-TAATGCACTCGAGAATGAAATCATACAAACTTAATAAC and TRE-HB022 5′-GCAGTAGATTCAGATCTCTAGGCTTCAATGCTAAGATGAG. The resulting PCR fragment was digested with XhoI/BglII (underlined) and cloned into SalI/BamHI, upstream of a yellow fluorescent protein (YFP) coding sequence in the same vector backbone as *At*TRE1-GFP. The sequence of each encoded fusion proteins was thoroughly checked by sequencing.

2.2. Transient and stable expression

The *At*TRE1-GFP and YFP-*At*TRE1 constructs were transiently expressed in tobacco leaves and analysed by confocal microscopy as described [18]. The YFP-*At*TRE1 construct was used also to transform tobacco bright yellow-cultured cells (BY-2), and also a cell line constitutively expressing the *Nicotiana plumbaginifolia* PMA2 H^+-ATPase fused to GFP [19]. A suspension cell culture was re-initiated from the selected (on 20 μg/ml hygromycin) resistant calli. The genetically encoded fluorescence was imaged from cultured-cells using an epifluorescent Leica DMR microscope (Wetzlar, Germany). The cells were plasmolysed by treatment for 5 min, prior to imaging, with 250 mM NaCl in Tris buffer (50 mM, pH 7.5). Cell turgor recovery of plasmolysed cells was achieved by diluting the salt to a final concentration of 10 mM with Tris buffer.

2.3. Yeast complementation assay

The cDNA of *At*TRE1 was PCR-amplified using the primers TRE-HB019 5′-TAATTCTAGAACAATGAAATCATACAAACTTAATAAC and TRE-HB020 5′-ATTATCTCGAGACCGGCTTCAATGCTAAGATGAG, and the product was directly cloned into PCR®8/GW/TOPO® (Invitrogen) according to the supplier's instructions. The resulting Gateway® compatible entry clone was recombined with the destination vector pYES-DEST52 (Invitrogen), to generate pYES-DEST52-AtTRE. The *AtTRE1* sequence in pYES-DEST52-AtTRE was checked by sequencing and contains a stop codon to prevent in-frame fusion to the V5 and 6xHis epitopes present in pYES-DEST52. Yeast strains used in this study were from the CEN.PK family and were described in [20]. The strain *ath1 ura* (MATa *MAL2-8c SUC2 ura3-52 ath1*Δ*::kanMX4*) was transformed with pYES-DEST-AtTRE essentially according to [21] using reagents purchased from Dualsystems Biotech (Zurich, Switzerland). Transformants were selected on solid minimal synthetic medium containing raffinose (2% w/v, Sigma) as carbon source and lacking uracil (MSD). Growth test on trehalose (2% w/v) was conducted with minimal synthetic growth medium without uracil, buffered to pH 4.8 with NaOH/succinate [20]. The induction of *At*TRE1 expression was achieved by preincubating the cells in MSD supplemented with 2% (w/v) galactose for 8 h.

2.4. Antibody preparation, protein extraction and analysis

A polyclonal antiserum was generated in rabbit against the *At*TRE1 peptides 93-RSYPKKQTPDPKSYI-107 and 377-SGCDFSTRWMRDPPNF-392 (Eurogentec, Seraing, Belgium). The anti-α-TIP (tonoplast intrinsic protein) was a gift from Dr. Liwen Jiang (Department of Biology, The Chinese University of Hong Kong), the anti-*At*Sec22 (At1g11890, a SNARE protein considered as an endoplasmic reticulum and Golgi membranes marker) was from Dr. Pierre Morsomme (University of Louvain, Belgium), a polyclonal anti-H^+-ATPase was obtained from Dr. Marc Boutry (University of Louvain, Belgium) [19,22]. Additional antibodies were purchased from Santa Cruz Biotechnology (California, USA). Total proteins were extracted from plant sample using the Plant CelLytic® reagent (Sigma) supplemented with plant proteases inhibitors cocktail (1% v/v, Sigma). Subcellular fractionation and purification of a plasma membrane-enriched fraction from Arabidopsis inflorescences was as described in [23]. All buffers were supplemented with 1% (v/v) plant proteases inhibitors cocktail as above. Subcellular fractionation of yeast cells and plasma membrane isolation was as described in [22]. Protein content was determined using the Bradford assay with bovine serum albumin as standard.

Yeast total proteins were obtained by boiling the cells for 5 min in 2× Laemmli sodium dodecyl sulphate (SDS) loading buffer containing the proteases inhibitors cocktail and glass beads. The proteins were separated in a 10% acrylamide gel, electroblotted on a polyvinylidene fluoride (PVDF) membrane, and immunodetected by chemiluminescence (GE Healthcare).

3. Results

The Arabidopsis genome as other plant genomes, encodes a single trehalase protein, *At*TRE1, the product of the gene At4g24040. The full-length cDNA encodes a polypeptide of 626 residues, with a calculated molecular mass of around 71 kDa. The protein contains 5 potential N-glycosylation consensus sites and the trehalase signatures PG[G]RFxExYxWDxY and QWDxPx[GAV]W[PAS]P present in the trehalase domain (residues 98–617). *At*TRE1 is predicted to harbour a putative transmembrane span (encompassing residues 46–63), absent in rice (*Oryza sativa*, OsTRE, Os10g37660, GID: 115482988) and soybean (*Glycine max*, GmTRE, GID: 4559292) homologues (see also the Supplementary figure for alignment and secondary structure prediction). To investigate the subcellular localisation of *At*TRE1 and the orientation of its catalytic domain (lumenal, cytoplasmic or extracellular), we first fused the protein to GFP at the C-terminus (*At*TRE1-GFP) and to YFP at the N-terminus (YFP-*At*TRE1). Both constructs, driven by a constitutive promoter were expressed transiently in tobacco leaf epidermal cells. For both fusion proteins, the resulting fluorescence outlined the periphery of the cell, suggesting an apoplastic localisation or a specific plasma membrane labelling (Fig. 1A and B). To distinguish between these possibilities, we stably expressed the YFP-*At*TRE1 construct into tobacco BY-2 cell. The imaged fluorescence was identical to what could be seen with cells expressing the *N. plumbaginifolia* H^+-ATPase PMA2, a plasma membrane marker fused to GFP (PMA-GFP) [19] (Fig. 1C and D). The two cell lines were subjected to plasmolysis. Fig. 1E and F shows for cells expressing respectively PMA-GFP or YFP-*At*TRE1 a shrinking of the protoplasm surrounded by a fluorescent plasma membrane connected to the cell wall by Hechtian strands. Fig. 1G shows YFP-*At*TRE1 fluorescence in a turgor-recovered cell.

To demonstrate that the endogenous *At*TRE1 is also plasma membrane-bound in Arabidopsis cells, we immunodetected the protein in a purified plasma membrane fraction. We first screened crude homogenate of different Arabidopsis organs for *At*TRE1 expression. *At*TRE1 (major band around 80 kDa) was highly expressed in flowers (Fig. 2A, lane F), 5-day old seedlings (lane S), and to some extent in green mature siliques (lane SI). The additional higher molecular mass bands

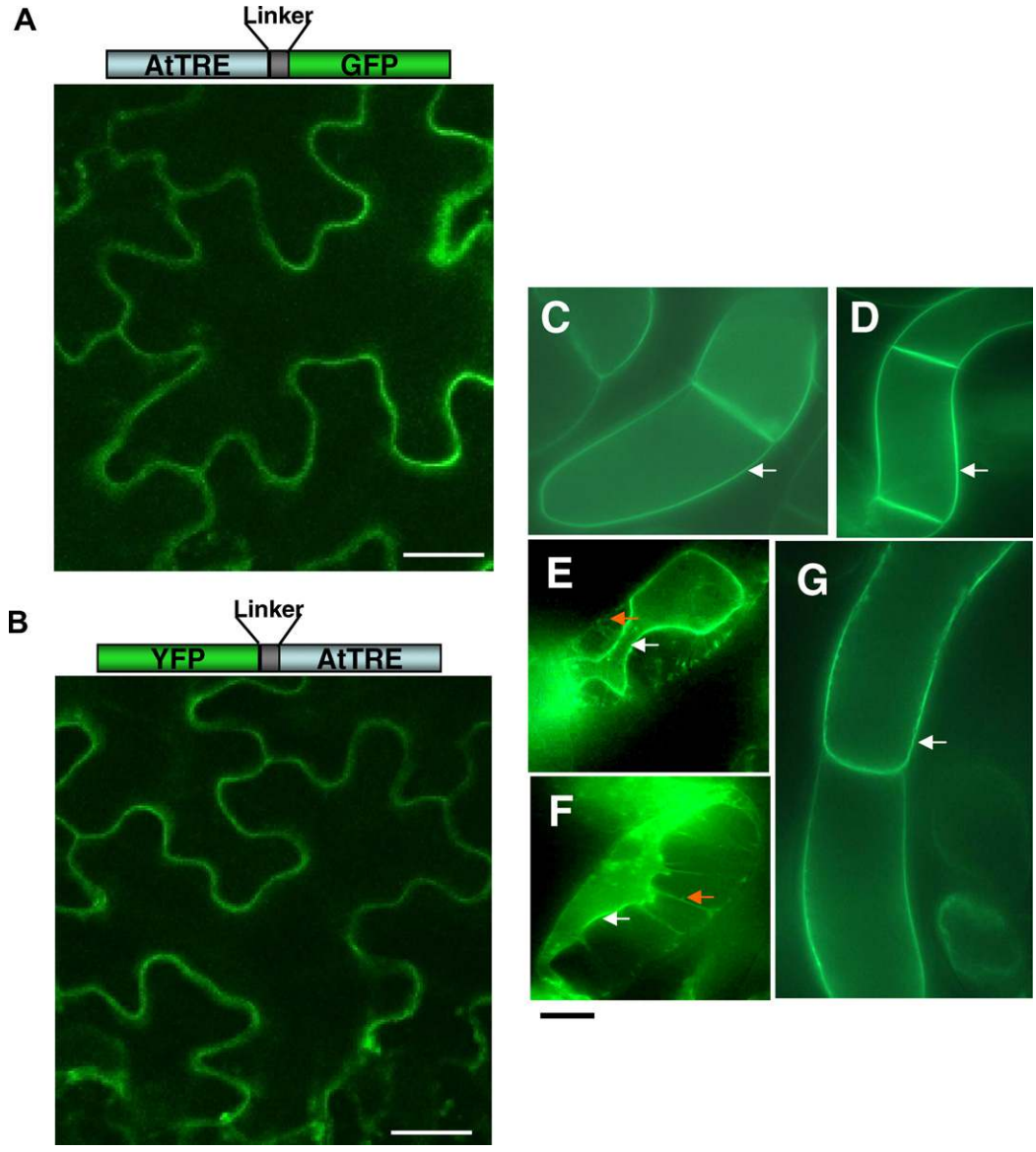

Fig. 1. Fluorescent protein tagged-*At*TRE1 is targeted to the plasma membrane of tobacco cells. (A) Confocal image of tobacco leaf epidermal cells expressing *At*TRE1 fused at its C-terminus to GFP. The cells were imaged 48 h after Agrobacterium-mediated transient transformation; fluorescence can be seen at the periphery of the cells, reminiscent of the outline of the plasma membrane. The white bar represents 20 μm. (B) Fluorescence of YFP-*At*TRE1 as in (A). (C and D) Localisation of PMA-GFP (C) and YFP-*At*TRE1 (D) in BY-2 cells. (E and F) Plasmolysed cells showing the shrinking of the protoplasm. Cells expressing YFP-*At*TRE1 (F) showed the same fluorescence pattern as those expressing PMA-GFP; the protoplasm is surrounded by the fluorescent plasma membrane (white arrowheads), the latter being attached to the cell wall by fluorescent Hechtian strands (orange arrowheads). (G) Turgor recovery after plasmolysis of YFP-*At*TRE1 expressing cell. The bar represents 20 μm and applies to panels (C)–(F).

may represent non-specific cross reacting antigens and/or post-translationally modified forms of *At*TRE1. None of these bands was seen with the preimmune serum (data not shown). We then used inflorescences of Arabidopsis to prepare microsomes followed by plasma membrane purification by phase partition. Equivalent amount of proteins from total microsomes (M) and the plasma membrane-enriched fraction (PM) were used to detect sequentially the enrichment of *At*TRE1 and H^+-ATPase in the two fractions. The data presented in Fig. 2B suggest that H^+-ATPase and *At*TRE1 are effectively enriched in the plasma membrane preparation from Arabidopsis inflorescences, in contrast to the tonoplast marker α-TIP and the ER/Golgi membrane marker *At*Sec22.

Having shown that *At*TRE1 is a plasma membrane-anchored protein, the next question we wanted to address was the orientation of the catalytic domain. We took advantage of yeast genetics and the knowledge available regarding the function and localisation of *Saccharomyces cerevisiae* trehalases. Since Ath1p is active extracellularly and is required for yeast growth on trehalose, we reasoned that if the catalytic domain of *At*TRE1 is apoplastic in plant cells, it may functionally replace Ath1p in yeast, although the two proteins are quite divergent. *At*TRE1 was expressed in *ath1* null yeast mutant strains under the control of the galactose inducible Gal10 promoter. The transformed cells, the wild-type and appropriate controls for auxotrophy and plasmid were analysed for complementation,

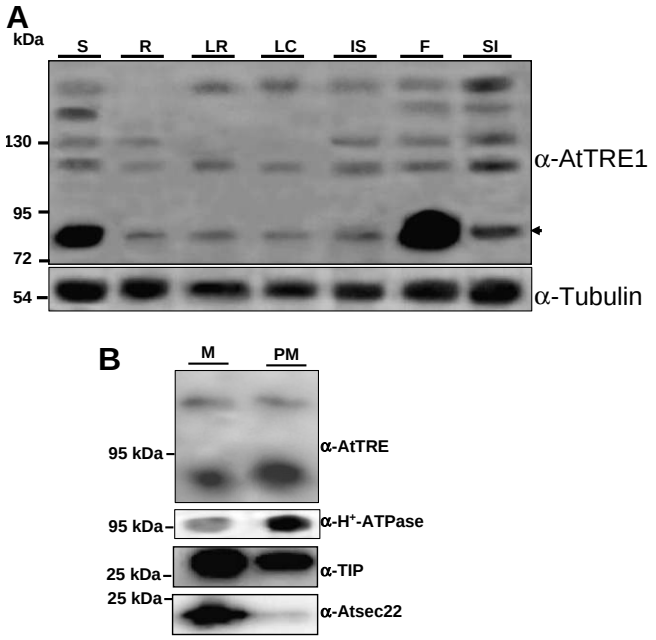

Fig. 2. *At*TRE1 is differentially expressed in plant organs and enriched in a plasma membrane fraction. (A) Western blot of 10 μg total proteins extracted from 5-day old Arabidopsis seedlings (S), roots of mature plants (R), rosette leaves of bolted plants (LR), cauline leaves (LC), inflorescence shoot (IS), flowers (F) and green siliques (SI). The blot was probed with anti-*At*TRE1 serum, the arrow indicates the major band detected of approximately 80 kDa. The lower panel shows the loading reference, anti-tubulin for each lane. (B) Western blot of plasma membrane-enriched microsomes from Arabidopsis inflorescences probed with anti-*At*TRE1 as in (A). The blot was reprobed with anti-H^+-ATPase (lower panel). The relative enrichment (PM/M) in plasma membrane was estimated by densitometry to be ~4 with respect to *At*TRE1 signal, and ~3.5 with respect to H^+-ATPase signal. The tonoplast marker α-TIP and the ER/Golgi membranes marker *At*Sec22, are not enriched in the plasma membrane fraction as compared to the microsomal fraction.

by assessing the growth on selective media adjusted at pH 4.8 and containing trehalose as the sole carbon source. Fig. 3A and B shows that under these growth conditions, provided *At*TRE1 was expressed, the potentially complemented strain (+*At*TRE) could grow comparably to the wild-type. We then checked whether the expressed *At*TRE1 in yeast was efficiently targeted to the plasma membrane using the anti-*At*TRE1 serum and subcellular fractionation. The antigen was detected in yeast total proteins extract (Fig. 4A), in crude microsome preparation (P15) but not in the supernatant containing cytosolic proteins (Fig. 4B). After plasma membrane purification by acid precipitation, *At*TRE1 was enriched in the plasma membrane fraction (PM) as compared to the endomembranes fraction (EM), paralleling the distribution of the yeast plasma membrane H^+-ATPase. We therefore concluded that *At*TRE1 expressed in yeast is targeted to the plasma membrane and can functionally substitute for Ath1p, in agreement with the hypothesised apoplastic orientation of its catalytic domain.

4. Discussion

Trehalase activity has been demonstrated in proteins extract of various plant species. The precise subcellular localisation of the protein in plant cell has not been demonstrated so far. Based on limited purification and enzymatic assay, and the prediction that the matured protein is glycosylated, it was assumed that plant trehalases may be soluble, secreted, and cell wall-associated. We have shown in this study that the only known Arabidopsis trehalase, *At*TRE1, is plasma membrane-bound in plant cell with its catalytic domain oriented towards the cell wall. Anchoring of *At*TRE1 to the plasma membrane is most likely due to the presence of a putative transmembrane span at the N-terminus which targets the protein to the secretory pathway (Figs. 1 and 2). The orientation of the catalytic domain in plant cell was extrapolated from functional analysis in yeast. *At*TRE1 expressed in yeast is plasma membrane-bound and can functionally replace the extracellular and unrelated Ath1p (Figs. 3 and 4), suggesting that its catalytic site is extracellular and capable of hydrolysing trehalose in this heterologous environment. Since the potential yeast H^+/trehalose symporter Agt1p is inactive at acidic pH [20,24], the growth observed on trehalose at pH 4.8 cannot be due to residual activity of the cytosolic Nth1p at this pH. According to the CaZy (Carbohydrate-Active Enzymes Server, http://afmb.cnrsmrs.fr/~cazy/CAZY/index.html) classification, Ath1p belongs to the glycosyl hydrolase family 65, with a tripartite catalytic domain. *At*TRE1, corresponding to about half the size of Ath1p, belongs to the glycosyl hydrolase family 37, which includes the human trehalase (TreH) [25] and the yeast cytosolic Nth1p [24,26]. The enzymatic properties, subcellular localisation and related mechanism of members of this *At*TRE1 containing – family may vary from one organism to another. Nth1p is cytosolic; TreH is post-translationally anchored to the plasma membrane of brush border cells via a GPI moiety at its C-terminus [25]. There is no consensus site for GPI modification in the available plant sequences.

The localisation and topology of *At*TRE1 suggest that its implication in the regulation of endogenous trehalose requires the substrate to be transported out of the cell. It has been shown that stressed *Escherichia coli* cells make use of their periplasmic trehalase to regulate the cytoplasmic level of trehalose by a futile cycle involving overproduction, excretion, and degradation to glucose, which is reutilised [27]. It is not yet clear whether plant cell can transport actively or passively trehalose in both directions across the plasma membrane. Alternatively, cytosolic trehalose level in plant cell may be regulated by an internal (endomembrane fraction en route to the plasma membrane) or internalised fraction of the enzyme. Even then, the substrate would have to cross a lipid bi-layer to reach the lumenal catalytic site. Another possibility may be that a soluble, non-related and yet uncharacterised isoform of the large plant glycosidase family may use trehalose as substrate. Feeding experiments suggest that exogenous trehalose is toxic to Arabidopsis and other trehalose non-accumulating plant species. The extracellular trehalose may be sensed as sucrose starvation [16], or may enter the cell and perturb the delicate balance between T-6-P and trehalose in favour of the latter, resulting in deregulation of sugar metabolism [15,16]. However, the sensitivity to exogenous trehalose may vary with the plant cell type. Indeed, it was shown that pollen grains from different plant taxa including tomato could germinate and form pollen tube on trehalose as the sole carbon source [28]. Phloridzin dehydrate, an inhibitor of glucose transport, depressed this germination on both trehalose and sucrose. Flowers and pollen grains in particular are enriched

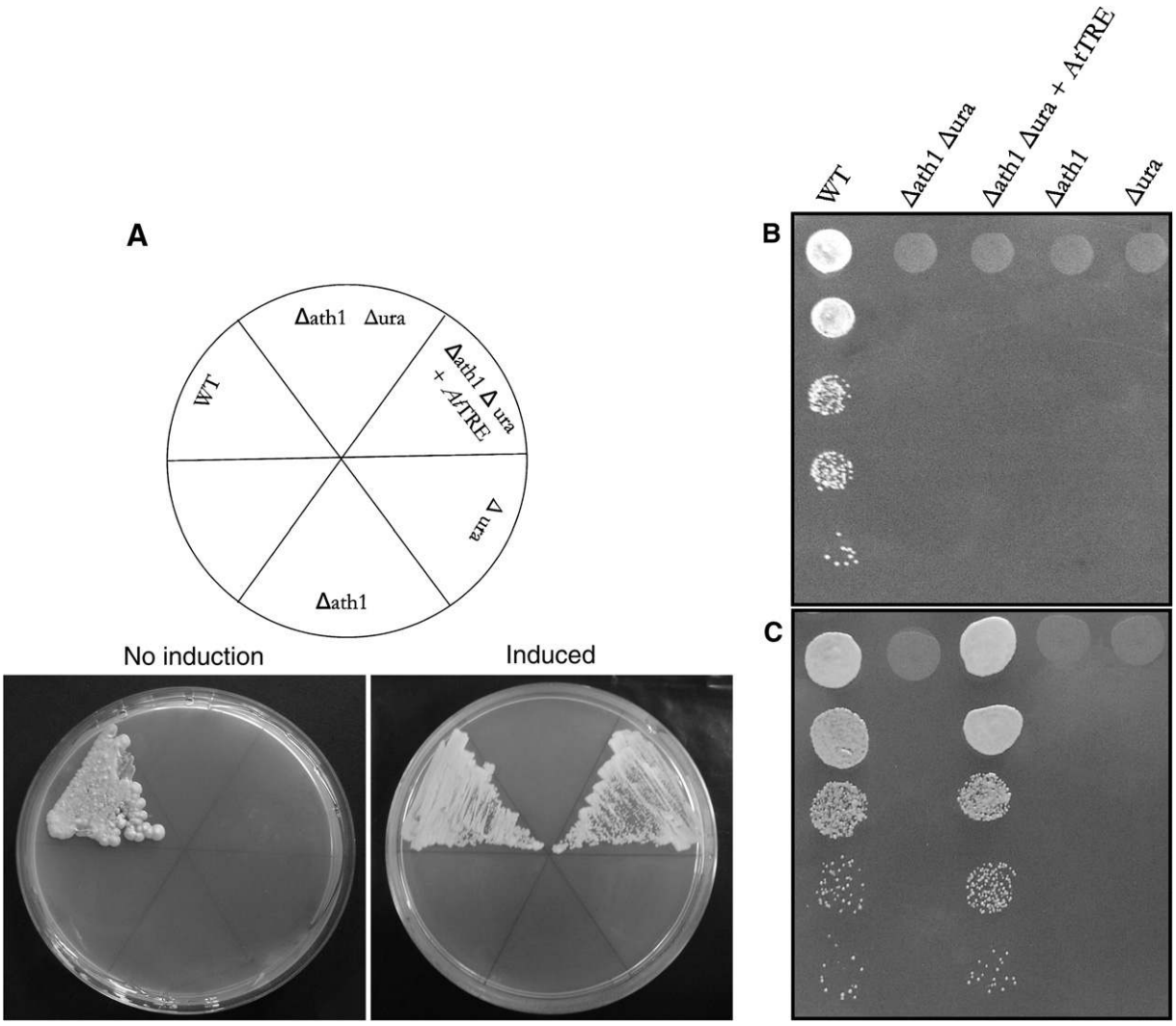

Fig. 3. The Arabidopsis *At*TRE1 can complement the lost of function of the yeast Ath1p. (A) Acidic growth (pH 4.8) on trehalose as the sole carbon source, on synthetic minimal medium without uracil; growth on trehalose requires either Ath1p (wild-type strain, WT), or *At*TRE1 (transformed *ath1* cells, +AtTRE, pre-galactose-induced plate on the right). Cells before induction (OD_{600} = 1), after induction (OD_{600} = 1.2). (B and C) A 10-fold serial dilution of cells prepared as in (A); without prior induction of AtTRE expression only the WT cells grow on trehalose (B); pre-incubation of transformed cells for 8 h in 2% (w/v) galactose allows the transformed *ath1* cells to grow on trehalose as the WT strain (C). The initial OD_{600} for each strain was adjusted to 1.2 before dilution. We checked that replacing the ccdB gene in pYES-DEST52 by a mock plant membrane protein did not complement the phenotype of *ath1* (data not shown).

in trehalase (Fig. 2A). It is possible that in vegetative tissues, in addition to a relatively low level of trehalase (Fig. 2A), the activity of the enzyme can be down-regulated by yet an unknown post-translational mechanism. Plant trehalase activity and transcripts seem to be up-regulated in vegetative tissues by biotic (pathogenic or symbiotic microorganisms [3,4]), and abiotic stress (drought, hypoxia [29,30]).

Although the transcript length of plant trehalase genes seems to be comparable (about 2.2 kb containing 10 exons), the primary structure of the encoded protein appears to vary from species to species. For instance, the full-length *At*TRE1 is 626 residues long as compared to 563 for OsTRE and 557 for GmTRE. More importantly, the rice and soybean protein lack the N-terminal extension of *At*TRE1 containing the potential transmembrane span, suggesting that their subcellular localisation may differ from that of *At*TRE1. Although the rice gene appears to encode two spliced variants, the variation concerns the size of the penultimate intron. ESTs analyses suggest that there is no splice variant for *At*TRE1. It was shown that a truncate *At*TRE1 (starting from the second methionine of the full-length protein), and lacking the putative transmembrane span can complement an *ath1* null mutant [10]. Although this truncated form may not enter the secretory pathway owing to the absence of the putative transmembrane span, the complementation of *ath1* phenotype by overexpressing the plant peptide suggest that it is somehow secreted. The N-terminus of this truncated form of *At*TRE1 may contain a cryptic signal peptide recognised by the yeast signal recognition system, therefore allowing the protein to be secreted. Alternatively and more likely, it may be that the strong promoter used to drive the expression in yeast resulted in some excretion of the mainly cytosol-localised protein, hence allowing the growth on trehalose of the transformed mutant strain. It is possible that OsTRE and GmTRE are soluble, secreted pro-

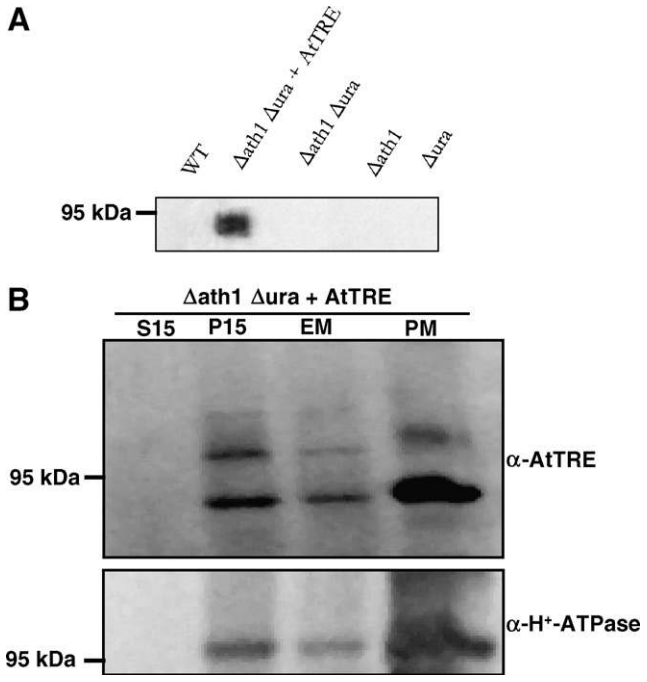

Fig. 4. The Arabidopsis trehalase expressed in yeast is plasma membrane-bound. (A) Western blot (10 μg of proteins) detection of *At*TRE1 from protein extract of control yeast strains and the transformed strain; the serum can only detect an antigen from extract of the transformed strain (after galactose-mediated induction). (B) The expressed *At*TRE1 in yeast cell is enriched in the purified plasma membrane fraction; S1: supernatant after microsomes sedimentation, P1: microsome fraction, EM: endomembranes after acid precipitation, PM: plasma membrane fraction obtained by acid precipitation. Anti-H^+-ATPase (lower panel) shows paralleled enrichment of this antigen in the tested fractions.

teins, but intriguingly, the available sequences also lack a signal peptide required for co-translational translocation into the ER lumen. As it was the case until recently for Arabidopsis, it is possible that the available OsTRE and GmTRE sequences are partial or not properly annotated.

Whether the function of *At*TRE1 and plant trehalase in general is limited to trehalose hydrolysis is not yet clear. Analysis of mutant alleles of the gene and post-translational regulation of the enzyme would shed more light on its importance in trehalose metabolism and beyond, in plant growth and development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.036.

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