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Cloning of Two Genes (*LAT1*,*2*) Encoding Specific L-Arabinose Transporters of the L-Arabinose Fermenting Yeast *Ambrosiozyma monospora*

Ritva Verho · Merja Penttilä · Peter Richard

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Abstract We identified and characterized two genes, *LAT1* and *LAT2*, which encode specific L-arabinose transporters. The genes were identified in the L-arabinose fermenting yeast *Ambrosiozyma monospora*. The yeast *Saccharomyces cerevisiae* had only very low L-arabinose transport activity; however, when *LAT1* or *LAT2* was expressed, L-arabinose transport was facilitated. When the *LAT1* or *LAT2* were expressed in an *S. cerevisiae* mutant where the main hexose transporters were deleted, the L-arabinose transporters could not restore growth on D-glucose, D-fructose, D-mannose or D-galactose. This indicates that these sugars are not transported and suggests that the transporters are specific for L-arabinose.

Keywords Sugar transport · L-Arabinose transport · L-Arabinose metabolism · Pentose fermentation · Metabolic engineering · Yeast

Introduction

L-Arabinose is an important carbon source for microorganisms. It is after D-xylose, the most widespread pentose sugar in our biosphere. Bacteria and fungi have different routes for the catabolism of this sugar. In bacteria, three different pathways are known. In the first two pathways, L-arabinose is oxidized to L-arabino γ-lactone, which is then cleaved by a lactonase to form L-arabonic acid. A dehydratase converts then L-arabonic acid to L-2-keto-3-deoxyarabonic acid. Here the two pathways start to differ. In one pathway, an aldolase cleaves L-2-keto-3-deoxyarabonic acid to pyruvate and glycoaldehyde [1]. In the other pathway, L-2-keto-3-deoxyarabonic acid is reacted to α-ketoglutaric semialdehyde and α-ketoglutaric acid by a dehydratase and a dehydrogenase, respectively [2]. A third pathway which is probably the most common in bacteria does not involve redox reactions. In this pathway, L-arabinose is converted to L-ribulose, L-ribulose 5-phosphate and D-xylulose 5-phosphate by L-arabinose isomerase, ribulokinase and L-ribulose 5-phosphate 4-epimerase, respectively [3]. In yeast and filamentous fungi, the L-arabinose is catabolised through a

R. Verho · M. Penttilä · P. Richard (✉)
VTT Technical Research Centre of Finland, Tietotie 2, Espoo, P.O. Box 1000, Fi-02044 VTT, Finland
e-mail: Peter.Richard@vtt.fi

pathway consisting of the five enzymes: aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase and xylulokinase. The metabolites are L-arabinitol, L-xylulose, xylitol, D-xylulose and D-xylulose 5-phosphate [4]. The genes coding for these enzymes were described in filamentous fungi [5–7]. In yeast and filamentous fungi, the reductions require NADPH while the oxidations require NAD [8, 9]. The yeast *Ambrosiozyma monospora* is unique in having an NADH-requiring L-xylulose reductase [9, 10]. The yeast *Saccharomyces cerevisiae* does not have an L-arabinose pathway; however, the redox neutral bacterial pathway as well as the fungal pathway have been functionally expressed is *S. cerevisiae* [11, 12]. The resulting strains were able to grow on L-arabinose indicating that L-arabinose is transported into the cell; however, it was suggested that this transport might be a limiting factor [12]. In bacteria, transport proteins for the L-arabinose transport (L-arabinose/H^+ symport) into the cell are well established, while knowledge on eukaryotic arabinose transporters is limited. For the yeast *Candida shehatae*, Lucas and van Uden [13] described an active L-arabinose transport, i.e. proton symport; however, no gene is associated to this activity. In *S. cerevisiae*, L-arabinose can be transported by the D-galactose transporter Gal2 [14], which is induced by D-galactose and repressed by D-glucose. Genes coding for specific L-arabinose transporters have not been described for eukaryotic microorganisms.

Material and Methods

Strains and Growth Conditions

The *Escherichia coli* strain DH5α was used in the cloning procedures. The *A. monospora* strain NRRL Y-1484 was used for cDNA synthesis and sugar transport measurement. The following *S. cerevisiae* strains were used: The CEN.PK was the starting material for all genetic constructions. The KY73 which had deletions in the hexose transporters *HXT1-7* and *GAL2* [15] was a gift of E. Boles. The strains H2707, H3188, H3189 and H3191 are based on the KY73. The H2707 expressed the *S. cerevisiae HXT1* from the pYX212 (R&D Systems) and was a gift of Dr. Anu Saloheimo. The strains H3188 and H3189 were transformed with the plasmids containing the *LAT1* and *LAT2*, respectively, which were obtained in the library screening as described in the following paragraph. The strain H3191 was transformed with pHL125 containing the *S. cerevisiae GAL2* gene [16], a gift of Dr. Richard Gaber. To test the growth on different carbon sources, different amounts of cells, 10^5, 10^4, 10^3, 10^2 and 10, were plated on selective medium with 2% of the sugars maltose, D-glucose, D-fructose, D-mannose or D-galactose.

The *S. cerevisiae* strain H2984 was used in the screening for improved growth on L-arabinose. The H2984 has the *Pichia stipitis XYL1* and *XYL2* genes encoding xylose reductase and xylitol dehydrogenase, respectively, and the endogenous *XKS1* encoding xylulokinase as described previously [10]. The genes *lad1* [17] and *lxr1* [18] from the mould *Hypocrea jecorina* coding for L-arabinitol 4-dehydrogenase and L-xylulose reductase, respectively, were integrated to the *TRP1* locus. The resulting strain was auxotroph for uracil, leucine and tryptophan. The *S. cerevisiae* strains were cultivated on selective medium with 2% D-glucose [19] and *A. monospora* on Yeast Nitrogen Base (Difco) with 4% L-arabinose.

Construction of a cDNA Library and Screening

The cDNA library was synthesised using the SuperScript cDNA synthesis kit (Invitrogen) essentially as described before [10] except that the cDNA was not ligated to the pEXP-

AD502 vector but to a modified pFL60 [20] vector which contains the URA3 for selection and to which the restriction sites SalI and NotI were added to the multiple cloning site. The H2984 described above was transformed with the cDNA library using the Gietz Lab transformation kit (Molecular Research Regents Inc.). About 500,000 transformants were plated on "Synthetic Complete," medium lacking uracil [19] with 2% D-glucose as carbon source. After cultivation at 30 °C, the cells were pooled and plated on a similar medium with 2% L-arabinose as sole carbon source. From the colonies that appeared after about 2 weeks, plasmids were rescued, transformed to *E. coli* and the cDNA sequenced.

L-Arabinose Uptake

The initial rate of L-arabinose uptake was measured using a modification of the method described by Bisson and Fraenkel [21]. A 10-μl aliquot of a sugar solution containing (1-^{14}C)-labelled L-arabinose (American Radiolabeled Chemicals Inc.) was incubated at 20 °C and was mixed with 40 μl of yeast suspension having the same temperature. After different time intervals 10 ml of cold (0 °C), 10 mM phosphate buffer at pH 7.0 was added, and the suspension was immediately filtrated using 25-mm-diameter Durapore filters (Millipore). The filter was washed with 10 ml of cold phosphate buffer. Filters were immediately transferred to 5 ml scintillation vials containing 3 ml OptiPhase "HiSafe" 3 Scintillation Cocktail (Perkin Elmer) and the radioactivity measured in a scintillation counter.

Results

Cloning of the L-Arabinose Transporter Genes

We constructed a cDNA library from *A. monospora*, one of the few yeast species which are known to ferment L-arabinose. The cells used as starting material for cDNA synthesis were harvested during growth on L-arabinose to ensure that it contained all the genes related to L-arabinose catabolism. The cDNA library in a yeast expression vector was transformed to a *S. cerevisiae* strain which contained all the genes of the fungal L-arabinose pathway, i.e., it was able to grow on L-arabinose, however at a very low rate. It grew so slowly that we were able to screen for improved growth on L-arabinose as previously described [10]. About 500,000 transformants were transferred to L-arabinose plates, and after about 2 weeks of cultivation, about 20 colonies were picked and plasmids extracted. Most of the plasmids contained the previously described NADH-dependent L-xylulose reductase [10]. Others contained two discrete open reading frames with sizes of about 1.6 kb. Sequencing of the cDNA revealed open reading frames with high homology to hexose transporters from other yeast species.

Measurement of the L-Arabinose Uptake Rate

The strains which were identified in the screening were tested for L-arabinose uptake, using radiolabelled L-arabinose. The control strain was the CEN.PK which is an *S. cerevisiae* haploid laboratory strain. In this strain at an L-arabinose concentration of 100 mM and 20 °C, the uptake rate was very close to the detection limit. The detection limit was estimated 0.05 nmol mg dry mass^{-1} min^{-1} under these conditions. The first of the two identified open reading frames with homology to hexose transporters resulted in L-arabinose uptake when expressed in *S. cerevisiae*. With 100 mM L-arabinose, the uptake rate was 0.2± 0.015 nmol mg dry mass^{-1} min^{-1} (Fig. 1a). We called this open reading frame *LAT1* for L-

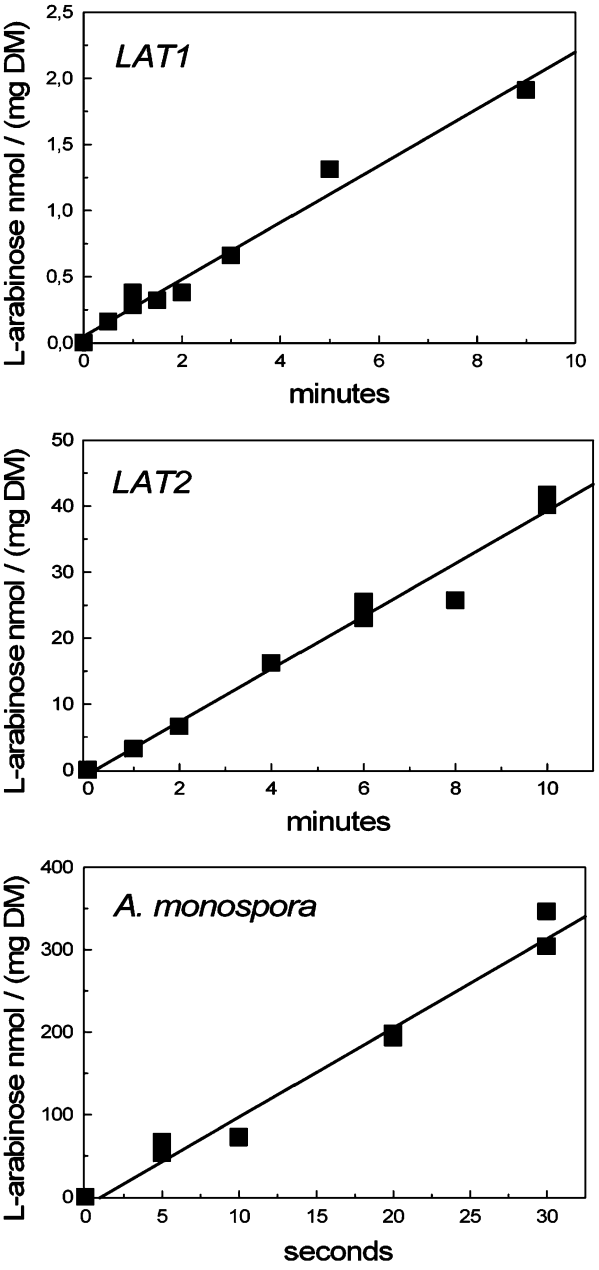

Fig. 1 L-Arabinose uptake in *S. cerevisiae* transformed with the *LAT1* or *LAT2* and in *A. monospora*. The uptake was measured at 20 °C and an L-arabinose concentration of 100 mM. *A. monospora* was grown on L-arabinose as the sole carbon source

arabinose transporter 1. The second open reading frame resulted also in L-arabinose uptake activity. The rate was 4±0.25 nmol mg dry mass^{-1} min^{-1} (Fig. 2b), and it was named *LAT2*.

The *LAT1* had an open reading frame of 1,557 bp, coding for a protein with 518 amino acids and a calculated molecular mass of 57,346 Da. The *LAT2* had an open reading frame

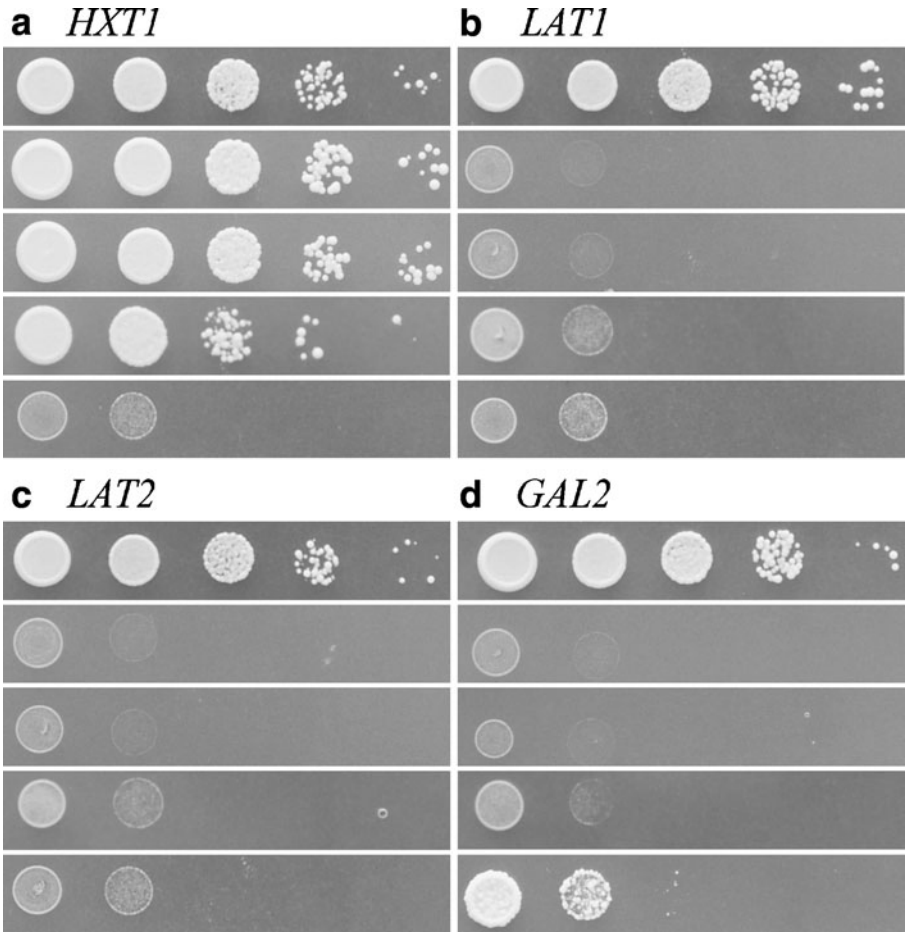

Fig. 2 Growth properties of the *hxt1-7 gal2* deletion strain expressing from a plasmid the *S. cerevisiae HXT1* gene (**a**), the *A. monospora LAT1* gene (**b**), the *A. monospora LAT2* gene (**c**) or the *S. cerevisiae GAL2* gene (**d**). Cells were plated in different concentrations on the carbon sources maltose, D-glucose, D-fructose, D-mannose and D-galactose (from *top* to *bottom*)

of 1,659 bp coding for a protein with 552 amino acids and a calculated molecular mass of 61,831 Da. The sequences were submitted to GenBank and have the accession numbers AY923868 and AY923869 for *LAT1* and *LAT2*, respectively.

To test how this uptake activity compared to the activity of the *A. monospora* strain from which the *LAT1* and *LAT2* were cloned, we measured the L-arabinose uptake after growing the strain on L-arabinose as a sole carbon source. The L-arabinose uptake was estimated to be 640±40 nmol mg dry mass^{-1} min^{-1} at an L-arabinose concentration of 100 mM at 20 °C.

Testing the Specificity

The *LAT1* and *LAT2* were expressed in a *S. cerevisiae* strain in which the hexose transporters required for growth on D-glucose, *HXT1-7*, and the galactose transporter required for growth on D-galactose, *GAL2*, were deleted. The strains were tested for growth on the carbon sources D-glucose, D-fructose, D-galactose, D-mannose and maltose (Fig. 2).

All strains were able to grow on maltose since maltose does not require any hexose transporter. The strains expressing the *LAT1* and *LAT2* were not able to grow on D-glucose, D-fructose, D-mannose nor D-galactose, indicating that *LAT1* and *LAT2* do not facilitate the transport of any of these sugars. As a control, we confirmed that the expression of *GAL2* could restore growth on D-galactose and the expression of *HXT1* restored growth on D-glucose, D-fructose and D-mannose.

Discussion

The yeast *S. cerevisiae* has no or only a very low L-arabinose uptake capacity. Under our conditions, when we measured the initial uptake of radiolabelled L-arabinose at 20 °C and an L-arabinose concentration of 100 mM, the parent *S. cerevisiae* strain had an uptake rate close to our detection limit which we estimated 0.05 nmol mg dry mass^{-1} min^{-1}. This is in good agreement with reports of Becker [22] and Kou et al. [14]. According to Kou et al., the L-arabinose transport is similar in non-D-galactose-induced cells and cells where the galactose transporter (*GAL2*) was mutated. A drastic increase in L-arabinose uptake was observed in D-galactose-induced cells which had also increased D-galactose transport activities. This suggested that the *GAL2* encodes a protein which facilitates the transport of D-galactose and L-arabinose. When the *GAL2* was expressed from a multi-copy plasmid, an elevated L-arabinose transport was shown by Becker [22]. At 30 °C and 10 mM L-arabinose, an uptake rate of 0.32 nmol mg dry mass^{-1} min^{-1} was observed.

The L-arabinose uptake is limiting the L-arabinose catabolism in recombinant *S. cerevisiae*. From the ethanol production rate, it was estimated that the L-arabinose uptake is probably not exceeding 0.08 nmol mg dry mass^{-1} min^{-1} [12]. However, the L-arabinose fermenting yeast *A. monospora* can take up L-arabinose at very high rates. For cells grown on L-arabinose, we estimated an uptake rate of 600 nmol mg dry mass^{-1} min^{-1} at 20 °C and 100 mM L-arabinose (Fig. 1c), i.e. *A. monospora* must have efficient L-arabinose transport proteins. We generated a cDNA library from *A. monospora* which was grown on L-arabinose to ensure the presence of mRNA from the genes required for L-arabinose catabolism. We used this cDNA library to screen in an *S. cerevisiae* strain which contained all the elements of the fungal L-arabinose pathway for improved growth on L-arabinose as described earlier [10]. The fact that we obtained L-arabinose transporters in this screen indicated that indeed the L-arabinose uptake is rate limiting in recombinant *S. cerevisiae*.

The transporters we obtained showed L-arabinose uptake rates, when expressed in *S. cerevisiae*, of 0.2 and 4 nmol mg dry mass^{-1} min^{-1}. This uptake rate was much lower than the uptake rate in *A. monospora*. There are several possible reasons for this difference. One reason might be that we did not identify the main transport protein in our screen; another reason might be that a posttranslational modification, which is absent in *S. cerevisiae*, is required for the activation. It is also possible that the membrane composition of *S. cerevisiae* does not support the optimal activity of the proteins or the transport protein is a protein complex which requires different subunits for optimal activity.

D-Xylose, another abundant pentose sugar, was taken up by *S. cerevisiae*. It was transported by the hexose transporters [23]. The sugar transporters *SUT1-3* from the yeast *P. stipitis* [24] transported D-xylose and D-glucose. Also D-xylose transporters identified in other yeast had transport activity with D-xylose and D-glucose [25]. D-Xylose and D-glucose have the same configuration from C1 to C4 in the Fischer projection. L-Arabinose and D-galactose also have the same configuration from C1 to C4, and they were also transported by the same transporter, the *GAL2*. The *LAT1* and *LAT2*, however, were different in that

respect that they did not transport pentose and hexose sugars but instead were specific for L-arabinose. D-Galactose was not transported neither was D-glucose, D-fructose or D-mannose. To our knowledge, this is the first report of eukaryotic sugar transporters which are specific for a pentose sugar such as L-arabinose.

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