Chapter 1
Utilization of α-Glucosidic Disaccharides by *Ogataea* (*Hansenula*) *polymorpha*: Genes, Proteins, and Regulation

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Abstract Utilization of α-glucosidic sugars such as maltose, maltotriose, isomaltose and sucrose has been extensively studied in a conventional yeast *Saccharomyces cerevisiae* as many important processes such as baking, brewing, and bioethanol production rely on fermentation of these sugars. In 1998, a non-conventional yeast *Ogataea* (formerly *Hansenula*) *polymorpha* was reported to grow on α-glucosidic disaccharides maltose and sucrose using intracellular α-glucosidase for their hydrolysis. Later on, the list of α-glucosidic sugars assimilated by *O. polymorpha* and hydrolyzed by its α-glucosidase was extended by adding maltotriose, isomaltose, palatinose, maltulose, and some others. In this chapter, we review the data on genetics, genomics, transport, and intracellular hydrolysis of α-glucosidic sugars in *O. polymorpha*. We also address evolution of yeast α-glucosidases and regulation of α-glucosidase and permease genes. Relevant data on other yeasts, mostly on *S. cerevisiae*, are used for comparison.

Keywords Maltose · Methylotrophic yeast · Sugar transport · Gene cluster · Genome mining

1.1 Introduction

Yeasts prefer sugars over other carbon sources and thrive in sugar-rich environment. In addition to glucose and fructose, many yeasts assimilate α-glucosidic oligosaccharides. α-Glucosidic oligosaccharides, maltose, isomaltose, and maltotriose (Fig. 1.1), emerge from degradation of starch and glycogen by amylases (Janecek 2009). Many plants (such as sugarcane and sugar beet) and berries contain a big amount of sucrose, a disaccharide of glucose and fructose. Sucrose is

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also synthesized by cyanobacteria and proteobacteria (Lunn 2002). Importantly, sucrose can be converted to isomers by enzymes of many organisms including plants, yeasts, filamentous fungi, bacteria, and even insects as shown in (Lee et al. 2011) and references therein. So, turanose, palatinose, and maltulose (Fig. 1.1) present in honey are isomerization products of sucrose. Palatinose is currently enzymatically produced from sucrose at a large scale and advertised as a novel healthy sugar with low glycemic index and no cariogenic effect (Sawale et al. 2017). Melezitose (Fig. 1.1) that is also present in honey is a main constituent of aphid honeydew (Daudé et al. 2012). Fig. 1.1 shows α-glucosidic sugars assimilated by a methylotrophic yeast *O. polymorpha* (Viigand 2018).

A question arises: Why do yeast communities have members that assimilate both methanol and disaccharides? The answer may lie in the natural habitat of these yeasts. For instance, the species of *Ogataea* have been isolated from spoiled orange juice, rotting plants, plant leaf surfaces and exudates, soil samples, and insect gut (Morais et al. 2004; Limtong et al. 2008; Naumov et al. 2017). These habitats certainly contain α-glucosidic sugars but may also contain methanol. In the soil, methanol is produced as a result of pectin and lignin degradation (Dorokhov et al. 2015). Furthermore, even living plant leaves emit methanol due to the recycling of pectin and lignin of the cell wall (Keppler et al. 2006). Thus, it is not surprising that yeast species metabolizing both disaccharides and methanol are found in nature.

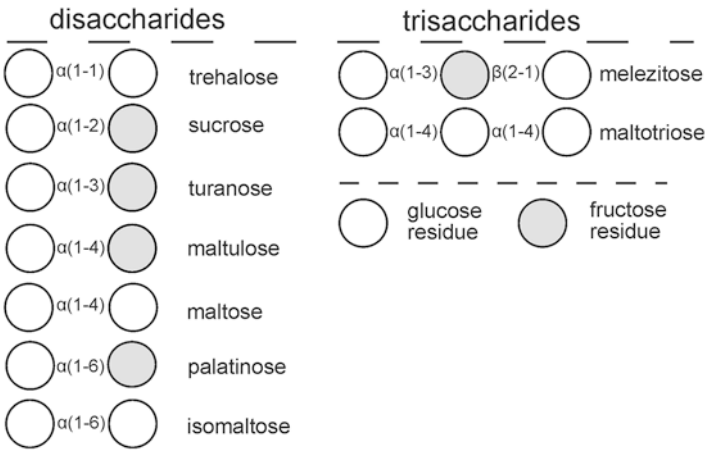

Fig. 1.1 α-Glucosidic di- and trisaccharides and the linkages between the sugar residues. (Viigand 2018)

1.2 *MAL* Clusters in Yeast Genomes

1.2.1 Saccharomyces cerevisiae

Metabolism of maltose has been thoroughly studied in *Saccharomyces* yeasts as they are commonly used in brewing – a process largely based on fermentation of maltose and maltotriose. Indeed, the beer wort produced by grain starch hydrolysis contains 50–60% of maltose and 15–20% maltotriose. During brewing, these oligosaccharides are transported into the yeast cell, hydrolyzed to glucose by maltases and isomaltases, and fermented to ethanol and CO_2 (Stewart 2016). In *S. cerevisiae*, the genes for maltose metabolism are genomically clustered comprising five *MAL* clusters, *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*, located near the telomeres of chromosomes VII, III, II, XI, and VIII (Brown et al. 2010; Charron et al. 1986; Needleman 1991; Vanoni et al. 1989). Each *MAL* cluster in *S. cerevisiae* consists of three genes (Fig. 1.2): *MALx1* (maltose permease gene), *MALx2* (maltase gene), and *MALx3* (*MAL*-activator gene) (Charron et al. 1986; Needleman 1991; Chang et al. 1988; Dubin et al. 1985). The "x" refers to the number of the cluster.

It should be noted that genomic clustering of functionally related genes is quite exceptional in eukaryotes. In addition to *MAL* clusters, gene clusters for utilization of galactose, allantoin, and nitrate are known in yeasts and filamentous fungi (Ávila et al. 2002; Kunze et al. 2014; Slot and Rokas 2010; Wong and Wolfe 2005).

1.2.2 Ogataea polymorpha

The maltase structural gene (*MAL1*) of *O. polymorpha* CBS 4732 was isolated from a genomic library of this strain in 2001 (Liiv et al. 2001). An open reading frame of 1695 bp encoding a 564 aa protein having 58% identity with *Candida albicans* maltase was characterized in the library clone p51 (Liiv et al. 2001). A scheme of the genomic insert of the p51 plasmid is shown in Fig.1.3.

Inspection of *O. polymorpha* CBS 4732 genomic library clones sequenced in the Génolevures project (Blandin et al. 2000; Feldmann 2000) identified several clones

Fig. 1.2 Composition of the *S. cerevisiae MAL3* cluster. *MAL31*, α-glucoside permease gene; *MAL32*, maltase gene; *MAL33*, *MAL*-activator gene. The lower panel depicts **position** of the *MAL* cluster (a dark vertical stripe) in chromosome 2 of *S. cerevisiae* S288C. Subtelomeric regions (50 kbp from the chromosome end) are shown in gray. (Viigand 2018)

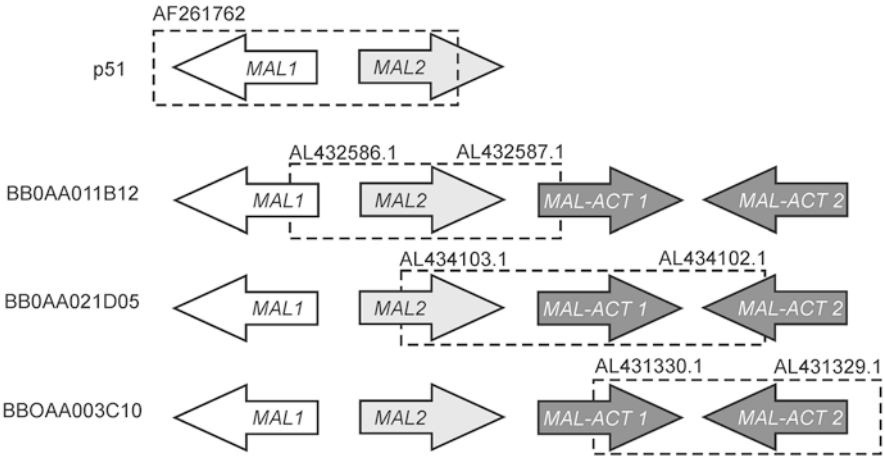

Fig. 1.3 Composition of the *O. polymorpha MAL* locus. Genomic insert in the Génolevures library clones is shown within the frame. Respective GenBank numbers of sequences belonging to *MAL* genes are shown above the frames. *MAL1* gene encodes a maltase (α-glucosidase), *MAL2* gene encodes an α-glucoside permease, and *MAL-ACT* genes encode putative *MAL*-activators. (Modified from Viigand 2018)

(BB0AA021D05, BB0AA011B12, and BB0AA003C10) (see Fig. 1.3) that contained fragments of *MAL* genes (Viigand 2018; Viigand et al. 2005).

Further sequencing of the Génolevures clone inserts revealed the composition of *O. polymorpha MAL* locus (Fig. 1.3). Similar to *S. cerevisiae*, a bidirectional promoter region was identified between the *MAL1* and *MAL2* genes of *O. polymorpha*. Two hypothetical transcriptional activator genes, *MAL*-activator 1 (*MAL-ACT 1*) and *MAL*-activator 2 (*MAL-ACT 2*), were detected next to the *MAL2* permease gene. Full-length sequence of the *MAL* locus of *O. polymorpha* CBS 4732 is accessible in the GenBank under number MH252366.

As the *MAL* loci of *S. cerevisiae* are subtelomeric, chromosomal location of the *O. polymorpha MAL* locus was inspected. The first *O. polymorpha* genome – of strain CBS 4732 – was sequenced in 2003 (Ramezani-Rad et al. 2003), but the sequence is not yet public. Therefore, the genome of *O. polymorpha* strain NCYC 495 leu1.1 (Grigoriev et al. 2014) was assayed instead. Comparison of genomic sequences of *MAL* clusters from *O. polymorpha* strains NCYC 495 leu1.1 and CBS 4732 revealed their identity, and the *MAL* cluster in *O. polymorpha* NCYC 495 leu1.1 was proven as not subtelomeric (Viigand 2018; Viigand et al. 2018).

Gene disruption experiments confirmed that *MAL1* and *MAL2* genes are, respectively, responsible for the hydrolysis and transport of α-glucosidic sugars in *O. polymorpha* (Viigand and Alamäe 2007; Viigand et al. 2016). Although hypothetical α-glucoside permease and α-glucosidase genes were recently detected outside the *O. polymorpha MAL* cluster, they were considered unnecessary for assimilation of α-glucosidic sugars (Viigand et al. 2018).

We conclude that *O. polymorpha* has a single *MAL* locus that consists of four genes coding for maltase MAL1 (syn. α-glucosidase, maltase-isomaltase), α-glucoside permease MAL2, and two putative MAL-activators. The functionality and role of *MAL*-activators have still to be clarified. However, there is indirect support of functionality for at least *MAL*-activator 1 gene. Namely, the promoter of this gene was regulated by carbon sources in the same way as the promoters of *MAL1* and *MAL2* genes (Viigand and Alamäe 2007). A four-gene non-telomeric *MAL* cluster similar to that of *O. polymorpha* was also detected in *O. parapolymorpha* which was formerly named *O. polymorpha* DL-1 (Viigand et al. 2018; Ravin et al. 2013).

1.2.3 Other Non-conventional Yeasts

Besides *O. polymorpha*, *MAL* clusters have been revealed in many yeasts such as *Lodderomyces elongisporus*, *Torulaspora delbrueckii*, *Meyerozyma guilliermondii*, *Cyberlindnera fabianii*, *Debaryomyces hansenii*, *Lipomyces starkeyi* (Viigand et al. 2018), *Scheffersomyces stipitis* (Jeffries and Van Vleet 2009), *Kluyveromyces lactis* (Fairhead and Dujon 2006; Leifso et al. 2007), and a filamentous fungus *Aspergillus oryzae* (Hasegawa et al. 2010). A recent genome mining study (Viigand et al. 2018) discovered the highest number of *MAL* clusters in *L. starkeyi* (Fig. 1.4), whereas no *MAL* clusters were found in *Blastobotrys adeninivorans* and *Schizosaccharomyces pombe*. Figure 1.4 illustrates the composition and number of *MAL* clusters in the genomes of non-conventional yeasts.

1.3 Transport of α-Glucosidic Sugars by *O. polymorpha*

Permeases responsible for maltose uptake have been experimentally characterized in *Saccharomyces* strains (Cheng and Michels 1989; Cheng and Michels 1991), *Torulaspora delbrueckii* (Alves-Araújo et al. 2004), *Candida utilis* (Peinado et al. 1987), *Schizosaccharomyces pombe* (Reinders and Ward 2001), and *O. polymorpha* (Viigand and Alamäe 2007).

The putative maltose permease gene *MAL2* from the *MAL* cluster of *O. polymorpha* was cloned in 2005. The MAL2 protein (582 amino acids) deduced from the respective gene had 51 and 57% identity to respective hypothetical maltose permeases of *Candida albicans* and *Debaryomyces hansenii* (Viigand et al. 2005). Inspection of genomes of a set of non-conventional yeasts thereafter revealed closer homologues of the MAL2: potential α-glucoside permeases of *O. parapolymorpha* (87% sequence identity to MAL2) and *Cyberlindnera fabianii* (67% identity to MAL2). Currently, the AGT1 protein of *S. cerevisiae* is the closest counterpart (37% sequence identity) of *O. polymorpha* MAL2 among experimentally studied permeases (Viigand et al. 2018). The *MAL2* disruption mutant of *O. polymorpha* lost the

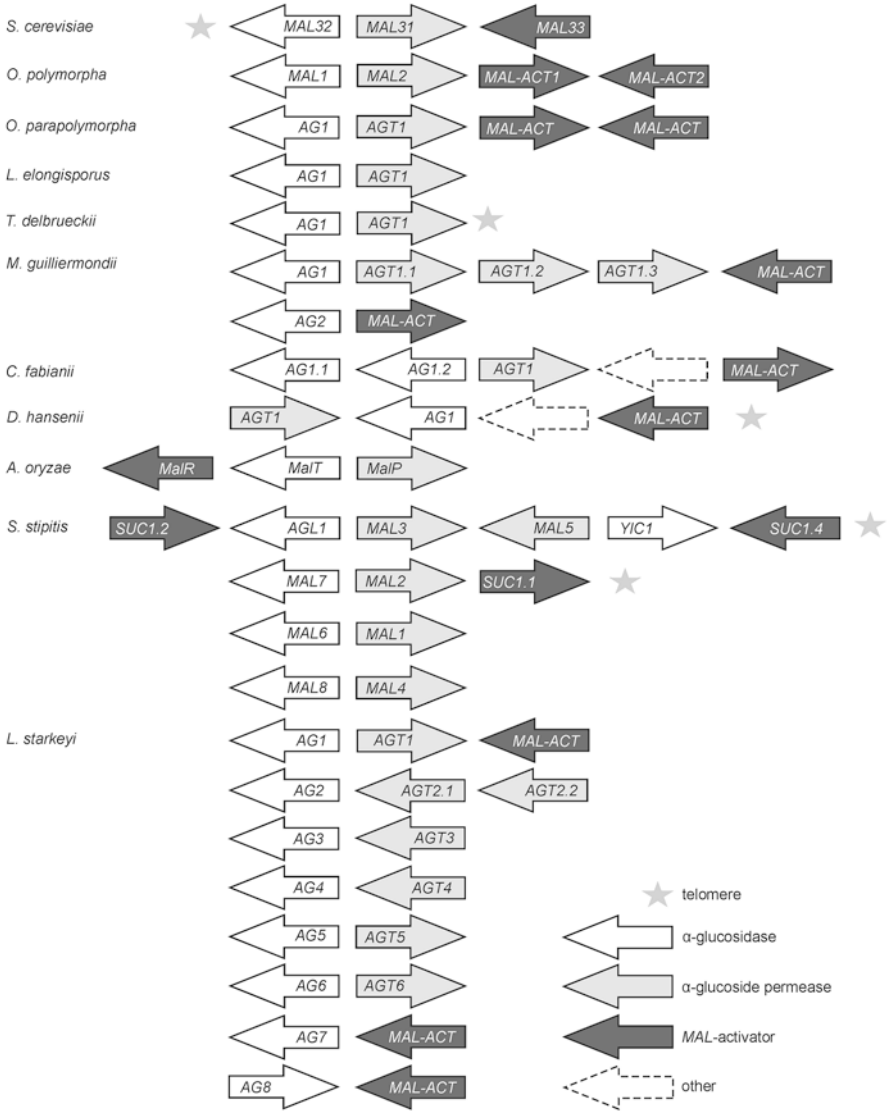

Fig. 1.4 *MAL* loci of some non-conventional yeasts (Viigand et al. 2018). Genes (potentially) encoding α-glucosidases (AG), α-glucoside transporters (AGT), and *MAL*-activators (*MAL*-ACT) are shown with different shading. Accession numbers and annotation data of the *AG* and *AGT* genes are given in Tables S1 and S2 of (Viigand et al. 2018)

ability to grow on maltose and sucrose, and complementation of the mutant with the *MAL2* gene on a plasmid restored the growth on these disaccharides (Viigand and Alamäe 2007). The MAL2 of *O. polymorpha* was also functional in *S. cerevisiae* (Viigand et al. 2005).

Similar to α-glucoside transporters of other yeasts (Reinders and Ward 2001; Stambuk et al. 2000; Stambuk and de Araujo 2001), the *O. polymorpha* MAL2 functioned as an energy-dependent proton symport (Viigand and Alamäe 2007). In agreement with that, the transport by MAL2 was highly sensitive to protonophores and energy uncouplers and had the highest activity at pH 5.0 (Viigand and Alamäe 2007).

Most α-glucoside transporters of *S. cerevisiae* (e.g., MAL61 and MAL31) have a narrow substrate range – they transport just maltose and turanose. In contrast to these transporters, the AGT1 permease of *S. cerevisiae* is highly promiscuous – it transports sucrose, maltose, turanose, PNPG (*p*-nitrophenyl-α-D-glucopyranoside), trehalose, α-methylglucoside, maltotriose, isomaltose, palatinose, and melezitose (Stambuk and de Araujo 2001; Han et al. 1995; Hollatz and Stambuk 2001; Day et al. 2002a). Notably, PNPG can be used as a chromogenic substrate to measure the activity of this type of permeases (Viigand and Alamäe 2007; Hollatz and Stambuk 2001). A recent study (Kulikova-Borovikova et al. 2018) shows that PNPG hydrolysis by the cells can also be used to evaluate permeabilization status of cell membrane in the case of PNPG-transporting yeasts (Kulikova-Borovikova et al. 2018).

In the first study (Viigand and Alamäe 2007), the MAL2 permease was reported to transport maltose, sucrose, turanose, trehalose, maltotriose, and PNPG. Further assay (Viigand et al. 2016) revealed that the MAL2 permease is also required for the growth of *O. polymorpha* on maltulose, melezitose, palatinose, and isomaltooligosaccharides. Because of the relaxed substrate specificity, the *O. polymorpha* MAL2 resembles the AGT1 of *S. cerevisiae* (Stambuk and de Araujo 2001; Han et al. 1995; Hollatz and Stambuk 2001) and should be considered an α-glucoside transporter. Even though the MAL2 is indispensable for the growth of *O. polymorpha* on trehalose (an α-1,1-linked disaccharide of glucose), the α-glucosidase (MAL1) protein does not hydrolyze this sugar (Liiv et al. 2001). It was shown later that for the hydrolysis of trehalose, *O. polymorpha* uses a specific enzyme – trehalase (Ishchuk et al. 2009).

The MAL2 transports PNPG with high affinity (K_m 0.51 mM) (Viigand and Alamäe 2007). The K_m value of the AGT1 permease of *S. cerevisiae* for PNPG is ~3 mM (Han et al. 1995). The MALx1 transporters from *S. cerevisiae* do not transport PNPG (Hollatz and Stambuk 2001). Inhibition of PNPG transport by various α-glucosidic substrates was used to evaluate affinity of the MAL2 permease of *O. polymorpha* for these substrates (Viigand and Alamäe 2007). Sucrose, maltose, trehalose, maltotriose, turanose, and α-methylglucoside (α-MG, also methyl-α-D-glucopyranoside) competitively inhibited the PNPG transport by MAL2 in *O. polymorpha* (respective K_i values were between 0.23 and 1.47 mM). However, though α-MG probably binds the MAL2 permease of *O. polymorpha*, this yeast does not grow on this synthetic sugar (Viigand et al. 2016). The *S. cerevisiae* AGT1 has lower affinity for its substrates than the MAL2 permease of *O. polymorpha*: K_m of the AGT1 for maltose is 5.1–17.8 mM (Stambuk and de Araujo 2001; Day et al. 2002b), K_m for sucrose is ~ 8 mM (Stambuk et al. 2000), and K_m for maltotriose is

4–18.1 mM (Stambuk et al. 2000; Day et al. 2002a). As the MAL2 permease of *O. polymorpha* has a considerably high affinity for α-glucosidic sugars, concentrations of these substrates are expected to be low in the habitat of *O. polymorpha*. Interestingly, the AGT1 protein of *S. cerevisiae* was also capable of glucose transport if overexpressed in a hxt1–17 gal2 deletion strain (Wieczorke et al. 1999).

The affinity of the MAL2 permease for maltose and sucrose is much higher than that of the maltase for these sugars (Viigand 2018; Liiv et al. 2001; Viigand and Alamäe 2007; Viigand et al. 2016), suggesting that these substrates must be concentrated into the cell to enable their efficient hydrolysis. The presence of intracellular substrate (e.g., maltose) is crucial for the induction of the *MAL* genes as reported for *O. polymorpha* (Viigand and Alamäe 2007) and *S. cerevisiae* (Wang et al. 2002). So, if a *MAL2* disruptant was incubated in the medium containing maltose, no maltase induction was recorded in the cells (Viigand and Alamäe 2007).

The AGT1 permease of *S. cerevisiae* was recently studied by Trichez et al. using mutagenesis (Trichez et al. 2018). Given that charged residues are usually responsible for sugar and proton binding by H^+-symporters, Trichez et al. selected four conserved charged residues in the predicted transmembrane α-helices 1 (Glu120, Asp123), 2 (Glu167), and 11 (Arg504) of the AGT1 permease for mutagenesis. Mutation of Glu120 and Arg504 to alanine most severely reduced the active transport of maltotriose and other α-glucosidic sugars by the yeast. In the case of Asp123 and Glu167, a double mutation (Asp123Gly/Glu167Ala) was required to reduce the transport (Trichez et al. 2018). Figure 1.5a shows alignment of *O. polymorpha* MAL2 transporter with AGT1 and MAL31 of *S. cerevisiae* in regions predicted to encode transmembrane domains (TMDs) 1, 2, and 11. Conserved charged residues proven crucial for active transport by the AGT1 are highlighted in the alignment, and predicted TMDs of MAL2 are shown overlined. Fig. 1.5b shows predicted structure of the *O. polymorpha* MAL2 permease. The charged residues of TMDs 1, 2, and 11 that are inferred to participate in active transport of α-glucosidic sugars are shown on dark background.

1.4 The MAL1 of *O. polymorpha* Among Yeast α-Glucosidases

The MAL1 protein of *O. polymorpha* was first characterized in 2001 (Liiv et al. 2001). This subchapter characterizes not only catalytic properties of the MAL1 protein but also its phylogenetic position among α-glucosidases of yeasts.

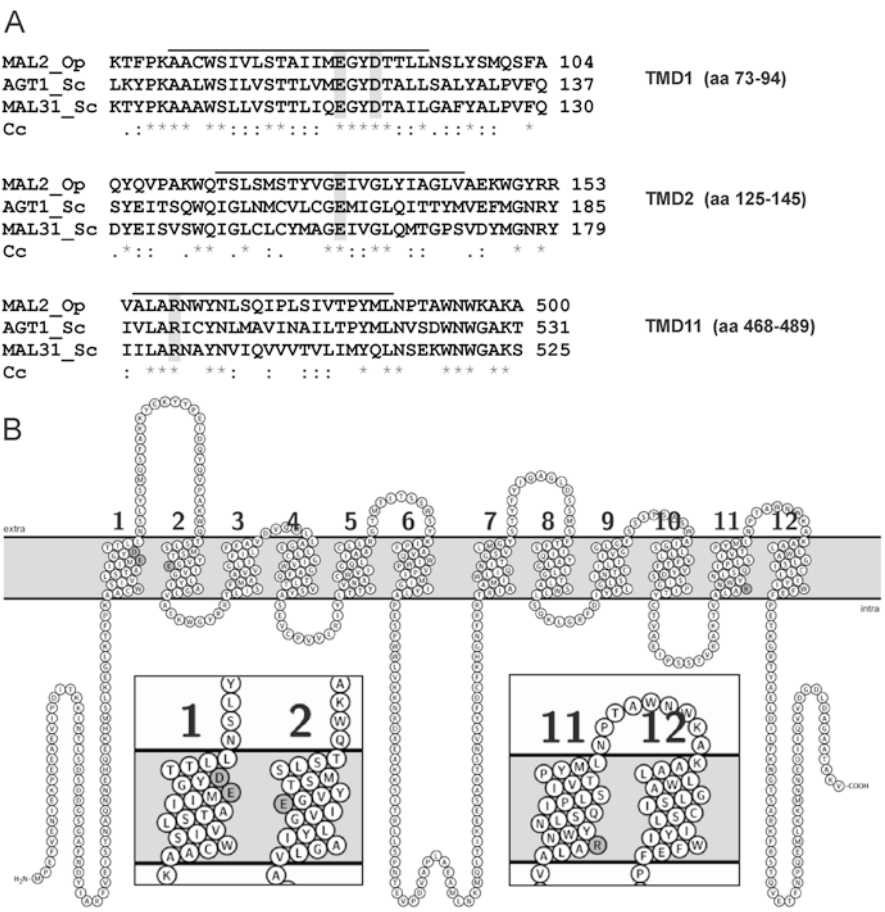

Fig. 1.5 (**a**) Extract of ClustalW alignment of the *O. polymorpha* MAL2 with AGT1 and MAL31 of *S. cerevisiae*. Sequences comprising the transmembrane domains (TMDs) 1, 2, and 11 are shown overlined. (**b**) Predicted TMDs of the MAL2 permease of *O. polymorpha*. The insert highlights charged residues of TMD1 (Glu87 and Asp90), TMD2 (Glu135), and TMD11 (Arg471) of *O. polymorpha* MAL2 assumed crucial for active transport of α-glucosidic sugars. TMDs were predicted through the CCTOP website (Dobson et al. 2015). The MAL2 permease was visualized using the Protter program (Omasits et al. 2014)

1.4.1 α-Glucosidases in CAZy

Yeast α-glucosidases belong to glycoside hydrolases (EC 3.2.1.–). Glycoside hydrolases (GHs) hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Lombard et al. 2014). For

example, the α-glucosidase MAL1 of *O. polymorpha* also hydrolyzes an α-glycosidic bond in α-MG and PNPG liberating methanol and *p*-nitrophenol from respective substrates (Liiv et al. 2001). Hydrolysis of a chromogenic substrate PNPG is widely used to quantitate catalytic activity of α-glucosidases (Liiv et al. 2001; Zimmermann et al. 1977). CAZy, a protein sequence-based database of Carbohydrate-Active enZymes, classifies maltases and isomaltases to family GH13 (Lombard et al. 2014). This family also includes α-amylases, pullulanases, neopullulanases, isoamylases, trehalose synthases, trehalose-6-phosphate hydrolases, and many others. The similarity of the amino acid sequences within the GH13 family proteins is low, yet they all share highly conserved regions and a catalytic machinery – three acidic catalytic residues located in conserved regions (Yamamoto et al. 2010). In the MAL1 protein of *O. polymorpha*, Asp199 was predicted as catalytic nucleophile, Glu257 as acid-base catalyst, and Asp338 as transition state stabilizer. The importance of Asp199 for the catalysis was experimentally proven: the Asp199Ala (D199A) mutant of MAL1 was catalytically inactive (Viigand et al. 2016).

1.4.2 Maltases and Isomaltases of Saccharomyces cerevisiae

S. cerevisiae has two types of α-glucosidases for the hydrolysis of α-glucosidic sugars: maltases (EC 3.2.1.20) and isomaltases (EC 3.2.1.10). Isomaltases have also been referred to as oligo-1,6-glucosidases, sucrase-isomaltases, and α-methylglucosidases. Maltases (e.g., MAL12 and MAL62) degrade maltose and so-called "maltose-like" sugars, maltotriose, sucrose, turanose, and maltulose, but are not able to degrade isomaltose and "isomaltose-like" sugars such as palatinose and α-MG (Viigand 2018; Needleman et al. 1978; Krakenaĭte and Glemzha 1983; Voordeckers et al. 2012). For composition and linkages in these sugars, see Fig. 1.1. For isomaltose degradation, *Saccharomyces* has isomaltases IMA1 to IMA5 (Naumoff and Naumov 2010; Teste et al. 2010). In addition to isomaltose, palatinose, and α-methylglucoside, isomaltases of *S. cerevisiae* also hydrolyze PNPG and sucrose (Teste et al. 2010; Deng et al. 2014).

1.4.3 Substrate Specificity of the O. polymorpha *MAL1*

Enzymatic assay of crude extract from *E. coli* cells expressing the MAL1 protein revealed maltose, sucrose, and α-methylglucoside as substrates for the enzyme, whereas trehalose, melibiose, and cellobiose were not hydrolyzed, indicating that the enzyme acts on α-1,4 (as in maltose) and α-1,2 (as in sucrose) glycosidic linkages (Liiv et al. 2001). In (Viigand et al. 2016), the substrate specificity of heterologously expressed and purified MAL1 protein was addressed, and additional substrates were revealed for the enzyme. It was shown that the MAL1 can hydrolyze the following maltose-like substrates with affinities decreasing in the order: maltulose, maltotriose, sucrose, turanose, maltose, and melezitose. From

Table 1.1 Substrate specificity of the *O. polymorpha* MAL1 (Viigand et al. 2016)

	Kinetic parameters		
Substrate	K_m (mM) ±SD	k_{cat} (1/min) ±SD	k_{cat}/K_m (mM/min)
Maltose-like substrates			
PNPG	0.52 ± 0.05	8476.4 ± 297.8	16300.7
Maltose	51.8 ± 3.7	10395.3 ± 297.8	200.7
Sucrose	25.1 ± 2.4	10984.2 ± 416.9	437.6
Turanose	34.0 ± 2.6	11864.3 ± 370.5	348.9
Maltotriose	20.2 ± 1.7	13597.9 ± 377.2	673.2
Maltulose	15.8 ± 0.8	2038.0 ± 33.1	129.0
Melezitose	115.0 ± 8.2	3870.9 ± 145.6	33.7
Isomaltose-like substrates			
α-Methylglucoside	35.5 ± 3.3	1806.4 ± 66.2	50.9
Isomaltose	27.3 ± 2.3	1191.1 ± 72.9	43.6
Palatinose	6.8 ± 0.4	1495.4 ± 26.5	219.9

SD standard deviation, *PNPG p*-nitrophenyl-α-D-glucopyranoside

isomaltose-like substrates, palatinose was the most suitable substrate, followed by isomaltose and α-MG. PNPG was the best substrate for MAL1 (Table 1.1).

Interestingly, the MAL1 protein could also hydrolyze fructooligosaccharides 1-kestose and 6-kestose (Viigand et al. 2016).This property has not been shown before for α-glucosidases. In the case of hydrolysis of trisaccharides melezitose and panose (see Fig. 1.1), the linkages hydrolyzed first by the enzyme were α-1,3 and α-1,6, respectively (Viigand 2018; Viigand et al. 2016). Though in early publications, the *O. polymorpha* MAL1 has been defined as a maltase, according to its substrate specificity, it rather belongs to maltase-isomaltases.

Phylogenetic analysis of yeast and fungal α-glucosidases revealed clustering of *O. polymorpha* MAL1 with α-glucosidases of *O. parapolymorpha*, *Cyberlindnera fabianii*, *Scheffersomyces stipitis*, *Lodderomyces elongisporus*, and *Meyerozyma guilliermondii* (Viigand et al. 2018). Of all these enzymes, only the *S. stipitis* MAL7, MAL8, and MAL9 (Viigand et al. 2018) and an α-glucosidase of *L. elongisporus* (Voordeckers et al. 2012) have been experimentally studied – these enzymes were reported to hydrolyze both maltose-like and isomaltose-like substrates. α-Glucosidases have been also studied in a phylogenetically old yeast *Schizosaccharomyces pombe*. The intracellular MAL1 protein of *S. pombe* hydrolyzed not only PNPG, maltose, and sucrose but also polymeric α-glucans dextrin and soluble starch (Chi et al. 2008). In addition to MAL1, *S. pombe* has an extracellular maltase AGL1, which was shown strictly specific for maltose – it did not hydrolyze other maltose-like sugars such as maltotriose and turanose (Jansen et al. 2006).

The malt extract and isomaltooligosaccharides were also substrates for *O. polymorpha* MAL1: the DP4 oligosaccharide was the longest substrate for the enzyme (Viigand et al. 2016). At the same time, the extracellular α-glucosidase of *S. pombe* used maltooligosaccharides with size up to DP7 – maltoheptaose (Okuyama et al.

2005). Some bacterial α-glucosidases also hydrolyze longer oligosaccharides (up to maltoheptaose) and in some cases also polysaccharides starch and dextrin (Egeter and Brückner 1995; Schönert et al. 1998, 1999; Cihan et al. 2011).

Similar to *S. cerevisiae* maltase (Kim et al. 1999), the MAL1 of *O. polymorpha* was very strongly inhibited by glucose and a diabetes drug acarbose. Fructose, a hydrolysis product of sucrose, turanose, maltulose, and palatinose (see Fig. 1.1), exerted much lower inhibiting power (Viigand et al. 2016). However, as in living yeast cells, glucose released from di- and trisaccharides is metabolized further; the in vivo inhibitory effect of glucose is probably lower than that recorded in vitro.

From yeast α-glucosidases, a three-dimensional structure has been resolved only for the *S. cerevisiae* isomaltase 1 (IMA1; PDB ID 3AJ7 and 3A4A). Structures of the IMA1 variants in complex with sugar ligands (maltose, isomaltose) have uncovered the amino acids bordering the active site (Y158, V216, G217, S218, L219, M278, Q279, D307, E411) with Val216 being crucial for selective binding of the substrate (Yamamoto et al. 2010, 2011; Deng et al. 2014). These nine positions vary between the maltases, isomaltases, and maltase-isomaltases (Voordeckers et al. 2012), and amino acids residing at these positions have been used as a signature in phylogenetic and substrate specificity analyses of α-glucosidases (Viigand et al. 2016, 2018; Voordeckers et al. 2012).

Table 1.2 illustrates signature amino acid patterns of α-glucosidases with known substrate specificity. α-Glucosidases hydrolyzing the α-1,6-glycosidic linkage have a Val residue at position equivalent to Val216 of IMA1 – a residue next to catalytic nucleophile Asp215. The corresponding residue of α-glucosidases that are able to hydrolyze maltose-like substrates is either Thr (Yamamoto et al. 2010) or in some cases Ala (Viigand et al. 2018; Tsujimoto et al. 2007). Respective residues are shown in bold in Table 1.2.

If Val216 of the *S. cerevisiae* IMA1 was replaced with a Thr; the enzyme gained the ability to hydrolyze maltose (Yamamoto et al. 2004). The *O. polymorpha* MAL1 has a Thr at a respective position (Table 1.2). Substitution of Thr200 with a Val drastically reduced the hydrolysis of maltose-like substrates by the *O. polymorpha* MAL1, whereas hydrolysis of isomaltose-like sugars was not affected (Fig. 1.6). Thus, it was concluded that Thr200 is required for efficient hydrolysis of maltose and maltose-like sugars. Indeed, the mutant Thr200Val became similar to isomaltases (Fig. 1.6).

1.4.4 The MAL1 of O. polymorpha *Is Ancient-Like*

Voordeckers et al. (Voordeckers et al. 2012) raised a hypothesis suggesting that modern maltases and isomaltases as those present in *S. cerevisiae* have evolved from a common promiscuous ancestor. The protein sequence of the ancestral protein (ancMalS) was predicted in silico, resurrected by heterologous synthesis in *E. coli*, and studied for enzymatic properties. Though the ancMalS was primarily

Table 1.2 Signature amino acids of α-glucosidases with known substrate specificity (Viigand et al. 2018)

α-Glucosidase	Signature amino acids (numbering as in *Sc* IMA1)									
	158	216	217	218	219	278	279	307	411	Substrate specificity
ancMALS	F	**T**	A	G	L	V	G	D	E	M-I
Le α-glucosidase	H	**T**	A	G	M	V	G	D	N	M-I
Op MAL1	F	**T**	A	G	L	V	G	D	N	M-I
Ss MAL7	F	**T**	A	G	L	V	G	T	N	M-I
Ss MAL8	Y	**T**	A	G	L	V	G	E	N	M-I
Ss MAL9	Y	**T**	A	G	M	V	G	E	N	M-I
Sc MAL32	F	**T**	A	G	L	V	A	E	D	M
Sp Mal1	Y	**A**	I	N	M	M	P	D	E	M
Ao MalT	I	**T**	V	N	M	L	P	D	D	M
Bs α-1,4-glucosidase	I	**A**	I	S	H	A	N	G	A	M
Sc IMA1	Y	**V**	G	S	L	M	Q	D	E	I
Sc IMA2	Y	**V**	G	S	L	M	Q	D	E	I
Sc IMA3/4	Y	**V**	G	S	L	M	R	D	E	I
Sc IMA5	F	**V**	G	S	M	V	G	S	E	I
An AgdC	F	**V**	I	N	F	M	P	D	D	I
Fo Foagl1	F	**V**	I	N	F	M	P	D	D	I
Bt oligo-1,6-glucosidase	V	**V**	I	N	M	T	P	D	E	I

M-I maltase-isomaltase, M maltase, I isomaltase. The amino acid at position corresponding to Val216 of *S. cerevisiae* IMA1 is shown in bold. AncMALS a resurrected hypothetical ancestor protein of *S. cerevisiae* maltases/isomaltases (Voordeckers et al. 2012), *Le Lodderomyces elongisporus*, *Sc S. cerevisiae*, *Ss Scheffersomyces stipitis*, *Sp Schizosaccharomyces pombe*, *Bs Bacillus stearothermophilus*, *Bt Bacillus thermoglucosidasius*, *Ao Aspergillus oryzae*, *Fo Fusarium oxysporum*, *An Aspergillus niger*

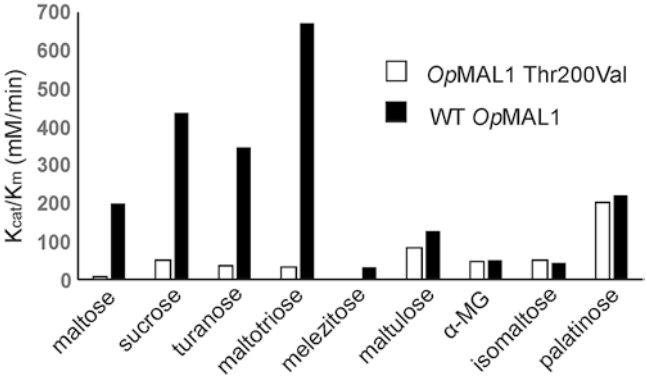

Fig. 1.6 According to catalytic efficiency k_{cat}/K_m (mM/min), a Thr200Val mutant of the *O. polymorpha* MAL1 protein is similar to an isomaltase. Compiled using data from Table 2 of (Viigand et al. 2016)

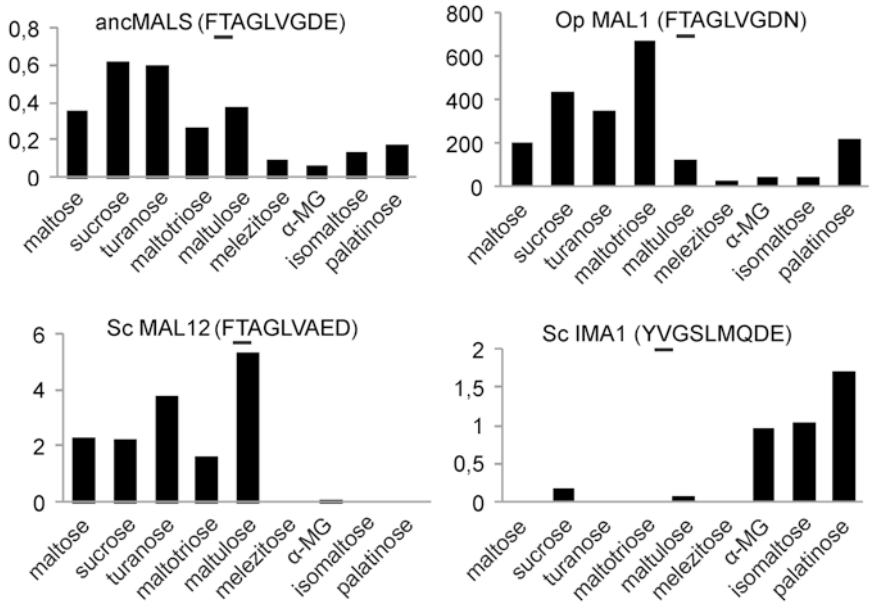

Fig. 1.7 Catalytic efficiencies, k_{cat}/K_m (mM/min), of the *O. polymorpha* (*Op*) maltase-isomaltase MAL1, ancestral maltase ancMALS, maltase MAL12 of *S. cerevisiae* (*Sc*), and isomaltase IMA1 of *Sc*. The signature amino acids of the enzymes (see Table 1.2) are presented in parentheses with the amino acid equivalent to Val216 of IMA1 shown underlined (Viigand et al. 2016)

active on maltose-like substrates, it also had a minor activity on isomaltose-like sugars (Voordeckers et al. 2012). The present-day α-glucosidases of *S. cerevisiae* preferentially hydrolyze either isomaltose-like sugars (IMA1, IMA2, and IMA5) or maltose-like sugars (MAL12, MAL32, MAL62) (Voordeckers et al. 2012); see Fig. 1.7 for MAL12 and IMA1).

Intriguingly, according to substrate specificity and the signature amino acids, the *O. polymorpha* MAL1 is highly similar to ancMALS (Table 1.2 and Fig. 1.7). Even though it has been claimed that both maltase and isomaltase activities cannot be fully optimized in a single enzyme (Voordeckers et al. 2012), the MAL1 properties confirm that it is still possible (Fig. 1.7).

When studying evolutionary origin of α-glucosidases, Gabriško (Gabriško 2013) noted close relatedness of fungal and bacterial enzymes suggesting bacterial ancestry of fungal α-glucosidases. In agreement with this suggestion, the maltase Mal1 of an "ancient" yeast *S. pombe* and a bacterial maltase (from *B. stearothermophilus*) both have an Ala and Ile at positions corresponding to respective amino acids Val216 and Gly217 of the *S. cerevisiae* IMA1 protein. The *S. cerevisiae* maltases have Thr and Ala at respective positions (Table 1.3). Interestingly, the *MAL1* gene of *O. polymorpha* has also a property of a bacterial gene – its promoter region is perfectly recognized in a bacterium *Escherichia coli* as it possesses two pairs of sigma 70-like sequences (Alamäe et al. 2003).

1.5 Regulation of the *MAL* Genes by Carbon Sources in *O. polymorpha*

1.5.1 General Features

Maltase synthesis in *O. polymorpha* is regulated by carbon sources in the growth medium: maltose and sucrose act as strong inducers, glucose as a repressor, and glycerol and ethanol allow moderate derepression (Alamäe and Liiv 1998). If *O. polymorpha* is grown on maltose or sucrose in the presence of a high concentration (2%) of glucose or fructose, induction of maltase is prevented. Interestingly, maltase activity is slightly repressed even during growth on maltose or sucrose if these substrates are provided at a high (2%) concentration. Therefore, glucose and fructose arising from the hydrolysis of maltose and sucrose in the cell most probably have some negative effect on the expression of *MAL* genes (Suppi et al. 2013).

Reporter gene assay of the bidirectional *MAL1-MAL2* promoter showed co-regulated expression in both directions, repression by glucose and induction by maltose, whereas the basal expression was higher in the direction of the permease gene (Viigand et al. 2005). It seems reasonable, because the permease activity is first required to provide intracellular maltose that is needed for induction of the *MAL* genes (Viigand and Alamäe 2007). Induction of the *O. polymorpha MAL1-MAL2* promoter by maltose (and sucrose) was stronger in the direction of maltase gene (Viigand and Alamäe 2007), and induced strength of the *MAL1* promoter was shown to constitute up to 70% of that of the *MOX* promoter (Alamäe et al. 2003). This knowledge can be used in biotechnological applications. The *MAL1* promoter has already been successfully used to overexpress and purify a biotechnologically relevant levansucrase protein from *E. coli* (Visnapuu et al. 2008).

1.5.2 Phosphorylated and Unphosphorylated Hexoses as Regulatory Signals

S. cerevisiae has three hexose kinases: hexokinase PI (HXK1) and PII (HXK2) that phosphorylate both fructose and glucose and a glucose-specific glucokinase (GLK1) (Zimmermann and Entian 1997). One of the hexokinases, the HXK2 of *S. cerevisiae*, has evolved to play a key role in establishing glucose repression (Gancedo 1998; Vega et al. 2016). Therefore, *S. cerevisiae* mutants defective in HXK2 lack glucose repression of many enzymes, including the maltase (Moreno and Herrero 2002; Zimmermann and Scheel 1977).

O. polymorpha has two hexose kinases: a hexokinase phosphorylating both glucose and fructose and a glucose-specific glucokinase (Kramarenko et al. 2000; Laht et al. 2002). Quite unexpectedly, hexokinase-negative mutants from chemical mutagenesis retained glucose repression of maltase synthesis losing only fructose repression (Kramarenko et al. 2000). Further assay of respective gene disruption mutants

(Suppi et al. 2013) confirmed that in contrast to *S. cerevisiae*, hexokinase has no specific role in establishing glucose repression in *O. polymorpha* – instead the ability of the cell to phosphorylate hexoses was required for downregulation of sugar-repressed genes, including the *MAL1*. In this report, glucose-6-phosphate was proposed as a signaling metabolite to trigger sugar repression (Suppi et al. 2013).

Highly intriguing results regarding the regulation of *MAL* genes were obtained through the study of double kinase-negative mutants of *O. polymorpha* (Suppi et al. 2013). As these mutants have no enzymes for glucose and fructose phosphorylation, they cannot grow on sugars (glucose, fructose, sucrose, maltose, etc.), but they grow perfectly on glycerol, methanol, and ethanol. Though not growing on sugars, these mutants are capable of sugar transport (Suppi et al. 2013). Importantly, when double kinase-negative mutants were cultivated on glycerol in the presence of glucose or fructose, a very high maltase activity was recorded in the cells (Suppi et al. 2013). Thus, sugars considered to be repressors of the *MAL* genes promoted their activation in these mutants. Based on these results, a hypothesis was raised according to which presence of disaccharides in the environment is sensed by *O. polymorpha* inside the cell through a transient increase of "free" glucose and fructose (Fig. 1.8). This signal is captured by a mechanism that is yet unknown and results in initial activation of the *MAL* genes (Viigand 2018; Suppi et al. 2013).

Indeed, *O. polymorpha* growing on non-sugar substrates (e.g., glycerol) is prepared to transport and hydrolyze maltose and sucrose – basal levels of both activities are detected in the cells (Viigand et al. 2005; Viigand and Alamäe 2007). In agreement with that, the maltase and maltose permease genes were, respectively, 89- and and 181-fold derepressed after the shift of *O. polymorpha* from glucose to methanol medium (van Zutphen et al. 2010). As the MAL1 protein has a very low affinity for disaccharides (Liiv et al. 2001; Viigand et al. 2016), its substrates should be concentrated in the cell by energy-dependent transport (Viigand and Alamäe 2007) to enable their efficient intracellular hydrolysis. Therefore, the hydrolysis reaction of disaccharides should produce a significant amount of free glucose (and fructose) inside the cell. As glucose- and fructose-phosphorylating activity in

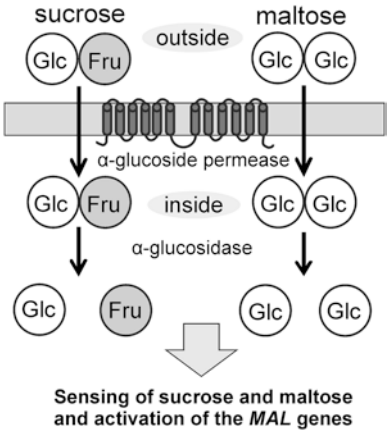

Fig. 1.8 Activation of the *MAL* genes in *O. polymorpha* by unphosphorylated glucose and fructose accumulating in the cell

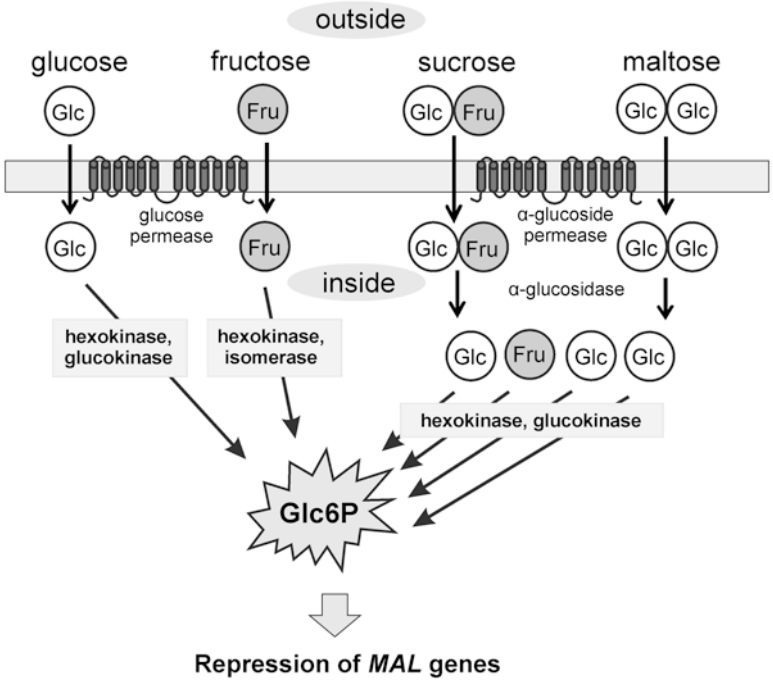

Fig. 1.9 Glucose-6-phosphate as a proposed repressing metabolite in the expression of *MAL* genes in *O. polymorpha*

O. polymorpha cells growing on gluconeogenic carbon sources is low (Kramarenko et al. 2000; Parpinello et al. 1998); at least part of glucose and fructose should stay unphosphorylated and trigger initial activation of the *MAL* genes.

Following internalization and hydrolysis of the disaccharides, the hydrolysis products are phosphorylated and channeled to glycolysis. As accumulation of phosphorylated derivatives of glucose and fructose causes repression of the *MAL* genes, a fine balance should exist between intracellular concentrations of free and phosphorylated species of monosaccharides, glucose, and fructose (Fig. 1.9). We assume that glucose-6-phosphate (Glc6P) signals for both glucose and fructose repression of *MAL* genes (Fig. 1.9).

Metabolic balance is also very important for *S. cerevisiae* growing on maltose. Therefore, if *S. cerevisiae* cannot manage intracellular metabolism of glucose resulting from maltose hydrolysis, excess of intracellular glucose will be exported by hexose transporters to prevent toxic effects exerted by high glucose concentration (Jansen et al. 2002).

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