Methylotrophic Yeasts: Current Understanding of Their C1-Metabolism and its Regulation by Sensing Methanol for Survival on Plant Leaves

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Abstract

Methylotrophic yeasts, which are able to utilize methanol as the sole carbon and energy source, have been intensively studied in terms of physiological function and practical applications. When these yeasts grow on methanol, the genes encoding enzymes and proteins involved in methanol metabolism are strongly induced. Simultaneously, peroxisomes, organelles that contain the key enzymes for methanol metabolism, massively proliferate. These characteristics have made methylotrophic yeasts efficient hosts for heterologous protein production using strong and methanol-inducible gene promoters and also model organisms for the study of peroxisome dynamics. Much attention has been paid to the interaction between methylotrophic microorganisms and plants. In this chapter, we describe how methylotrophic yeasts proliferate and survive on plant leaves, focusing on their physiological functions and lifestyle in the phyllosphere. Our current understanding of the molecular basis of methanol-inducible gene expression, including methanol-sensing and its applications, is also summarized.

Introduction

Methylotrophic yeasts are capable of utilizing methanol as the sole source of carbon and energy. These yeasts belong to a restricted number of genera, including *Komagataella*, *Ogataea*, *Kuraishia*, and *Candida* (Kurtzman, 2005; Péter *et al.*, 2005; Suh *et al.*, 2006; Limtong *et al.*, 2008), while methylotrophic bacteria belong to diverse genera and subclasses (Kolb, 2009). Methylotrophic yeasts can also use another one-carbon (C1) compound, methylamine, as a nitrogen source, but not as the sole carbon source.

Since the first isolation of the methylotrophic yeast *Kloeckera* sp. (later identified as *Candida boidinii*) in 1969 (Ogata *et al.*, 1969), both their physiological functions and their applications have been intensively studied. During the 1970s, the metabolic pathways for methanol assimilation and dissimilation were elucidated mainly with *Candida boidinii* and *Hansenula polymorpha* (reclassified as *Ogataea polymorpha*), and methylotrophic yeasts were revealed to have a common methanol-utilizing pathway (Anthony, 1982; Veenhuis *et al.*, 1983; Tani, 1984; Yurimoto *et al.*, 2002). One of the differences between bacterial and yeast methanol metabolism is in the initial oxidation reaction of methanol. While methylotrophic bacteria oxidize methanol to formaldehyde by using a pyrroloquinoline quinone (PQQ)- or NAD^+-dependent dehydrogenase, methylotrophic yeasts oxidize methanol with an alcohol oxidase (AOD) using molecular oxygen as an electron acceptor. When methylotrophic

yeasts grow on methanol, genes encoding a number of enzymes and proteins involved in methanol metabolism are strongly induced. This strong inducibility by methanol together with the ability to grow to extremely high cell density have enabled methylotrophic yeasts to become promising hosts for high-level heterologous protein production. During the mid-1980s to early 1990s, heterologous gene expression systems driven by strong methanol-inducible gene promoters were developed in various methylotrophic yeast strains, including *Pichia pastoris* (reclassified as *Komagataella phaffii*), *H. polymorpha*, and *C. boidinii* (Cregg *et al.*, 2000; Gellissen, 2000; Yurimoto, 2009). Another characteristic of yeast methanol metabolism is that methylotrophic growth is accompanied by massive development of a membrane-bound organelle, the peroxisome, in which AOD and other key methanol-assimilating enzymes are compartmentalized. Therefore, methylotrophic yeasts have also been used as model organisms to reveal the molecular machineries and mechanisms of peroxisomal protein import as well as peroxisome degradation by selective autophagy (pexophagy) (van der Klei *et al.*, 2006; Oku *et al.*, 2010).

Methanol is a volatile atmospheric carbon compound, and its annual emission is estimated to be 100 Tg (Galbally *et al.*, 2002). Methanol, which originates from methylesters in the plant cell wall constituent pectin, is emitted from plant leaves during plant cell elongation and division (Nemecek-Marshall *et al.*, 1995; Fall *et al.*, 1996). The phyllosphere, defined as the aerial portion of plants, has been recognized as a habitat for methylotrophic microorganisms. Phyllospheric methylotrophic bacteria were identified in the 1980s (Corpe *et al.*, 1982), and since then, the symbiotic relationship between plants and methylotrophic bacteria, *Methylobacterium* spp., which can promote plant growth, has been intensively investigated (Vorholt, 2012; Dourado *et al.*, 2015). Similarly, a number of methylotrophic yeast strains have been isolated from plant resources, e.g. forest soils, fallen leaves, and the skins of olives and grapes (Limtong *et al.*, 2008; Péter *et al.*, 2009). However, the ecology and physiology of methylotrophic yeasts in the phyllosphere have not been studied in detail.

In this chapter, after outlining methanol metabolism in methylotrophic yeasts, we describe how methylotrophic yeasts utilize carbon and nitrogen sources in the phyllosphere by regulating their cellular metabolism. Then the recently revealed molecular basis of methanol-inducible gene expression, including the methanol-sensing machinery is summarized.

Methanol metabolism in the methylotrophic yeasts

Fig. 11.1 summarizes the pathway of methanol metabolism, which so far is common to all isolated methylotrophic yeasts (Yurimoto *et al.*, 2002, 2011). Methanol metabolism begins with the oxidation of methanol to formaldehyde by AOD. AOD is a flavoprotein that contains FAD and uses O_2 as an electron acceptor to yield formaldehyde and H_2O_2, both of which are extremely toxic to cells. H_2O_2 is broken down by catalase (CTA) and peroxiredoxin (Pmp20) (Horiguchi *et al.*, 2001a,b). Formaldehyde, which is situated at the branch point between assimilation and dissimilation pathways, is a key central intermediate in methanol metabolism (Yurimoto *et al.*, 2005). In the assimilation pathway, formaldehyde (C1-compound) is fixed to xylulose 5-phosphate (Xu5P; C5 compound) by dihydroxyacetone synthase (DAS) to generate two C3 compounds, dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate (GAP). DHA is phosphorylated by dihydroxyacetone kinase (DAK) to yield dihydroxyacetone phosphate (DHAP). GAP and DHAP are used for the synthesis of cell constituents and the regeneration of Xu5P after rearrangement reactions.

In the cells of methylotrophic yeasts, peroxisomes, the organelles specialized for methanol metabolism, massively proliferate during growth on methanol. The peroxisomal localization of AOD, CTA, Pmp20, and DAS has been described in detail. Recently, proteomic analysis of the peroxisomal fraction of methanol-grown *P. pastoris* cells revealed that further assimilatory metabolism catalysed by DAK and the sugar phosphate rearrangement reactions are also localized within peroxisomes (Russmayer *et al.*, 2015).

In the dissimilation pathway, formaldehyde is oxidized to CO_2 by the glutathione-dependent formaldehyde oxidation pathway. Formaldehyde reacts non-enzymatically with the reduced form of glutathione (GSH) to form *S*-hydroxymethyl

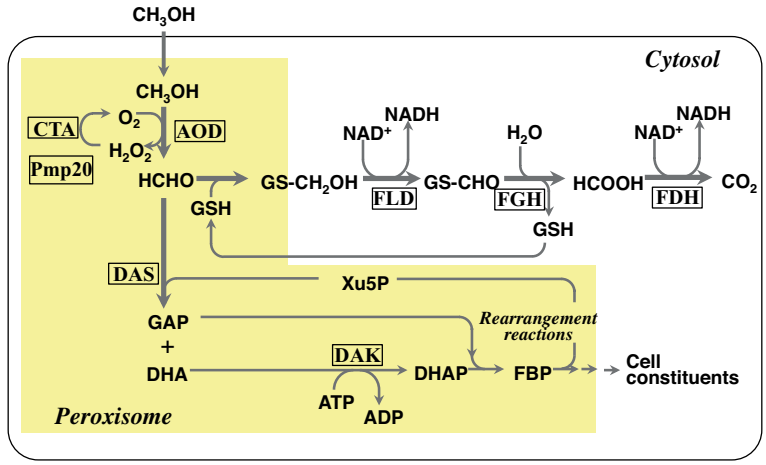

Figure 11.1 Methanol metabolism in methylotrophic yeasts. AOD, alcohol oxidase; CTA, catalase; DAK, dihydroxyacetone kinase; DAS, dihydroxyacetone synthase; FDH, formate dehydrogenase; FGH, *S*-formylglutathione hydrolase; FLD, formaldehyde dehydrogenase; Pmp20, peroxiredoxin (glutathione peroxidase). Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; GS-CH2OH, *S*-hydroxymethyl glutathione; GS-CHO, *S*-formylglutathione; GSH, reduced form of glutathione; Xu5P, xylulose 5-phosphate. Reactions occurring within peroxisomes are highlighted in yellow.

glutathione (*S*-HMG). Since GSH was shown to be present in peroxisomes, *S*-HMG can be formed within peroxisomes and then be exported to the cytosol (Horiguchi *et al.*, 2001b; Yurimoto *et al.*, 2003). *S*-HMG is oxidized to *S*-formylglutathione (*S*-FG) by NAD^+-linked and GSH-dependent formaldehyde dehydrogenase (FLD). *S*-FG is then hydrolysed to formate and GSH by *S*-formylglutathione hydrolase (FGH). Finally, formate is oxidized to CO_2 by NAD^+-linked formate dehydrogenase (FDH). Therefore, in methylotrophic yeasts, the formaldehyde oxidation pathway is physiologically responsible not only for formaldehyde detoxification but also for energy generation (Sakai *et al.*, 1997; Lee *et al.*, 2002; Yurimoto *et al.*, 2003).

Survival strategy in the phyllosphere: nutrient sources for methylotrophic yeasts

Plants emit various volatile organic compounds. Among them, 100 Tg of methanol per year is estimated to be emitted from plants (Galbally *et al.*, 2002; Laothawornkitkul *et al.*, 2009), and the main origin of methanol is thought to be the methylester residue of pectin, a major constituent of plant cell walls (Fall *et al.*, 1996). The above-ground part of plants, termed the phyllosphere, is one of the main habitats of methylotrophic microorganisms. Indeed, methylotrophic yeasts can often be isolated from plant material (Limtong *et al.*, 2008; Péter *et al.*, 2009). However, while the symbiotic relationship between plants and methylotrophic bacteria has been well characterized (Vorholt, 2012), it was not known whether methylotrophic yeasts can survive and proliferate in the phyllosphere.

Methanol emission from the phyllosphere was first reported by Nemecek-Marshall *et al.* (1995). In this report, methanol was detected in the atmospheric phase of a gas chamber in which a leaf of a growing plant was compartmentalized. Methanol was thought to be emitted from stomata because methanol emission was correlated with diurnal stomatal opening (Nemecek-Marshall *et al.*, 1995). However, it does not seem likely that phyllospheric microorganisms directly utilize gas-phase methanol. And until recently it was also unclear how much methanol is present in the phyllosphere and available to methylotrophic microorganisms.

In order to address these questions, we developed a cell-based methanol assay system using *C. boidinii* cells expressing the yellow fluorescent protein Venus tagged with peroxisome targeting signal 1 (Venus-PTS1) under the control of the methanol-inducible *DAS1* promoter, and

established a method to quantify methanol concentration on the surface of plant leaves (Kawaguchi *et al*., 2011). The *DAS1* promoter was chosen because it is the strongest methanol-inducible promoter and the level of methanol-independent expression (derepression) with the *DAS1* promoter is lower than that of the *AOD1* promoter (Yurimoto *et al*., 2000). The methanol sensor cells were inoculated onto the leaves of *Arabidopsis thaliana*, and the fluorescence intensity of the cells was determined after a 4-h incubation. When cells were inoculated onto young leaves (grown 2–3 weeks after germination), the estimated methanol concentration on the leaves changed periodically by 0–0.2% (ca. 0–60 mM): higher in the dark period (25–60 mM) and lower in the light period (0–5 mM). This daily oscillation in methanol concentration was opposite from previous observations, which indicated that atmospheric methanol was higher in the light period during stomatal opening (Nemecek-Marshall *et al*., 1995). Methanol, which accumulated in the spongy parenchyma of the leaf during stomatal closing in the dark period, diffuses to the surface of the leaf. In addition, quantitative transcript analyses revealed that the expression of methanol-inducible genes, such as *AOD1* and *DAS1*, was higher in the dark period and lower in the light period, corresponding to the methanol concentration in the phyllosphere (Kawaguchi *et al*., 2011).

We confirmed that the methylotrophic yeasts *C. boidinii* and *P. pastoris* could proliferate on the leaf surface of growing *A. thaliana* (Kawaguchi *et al*., 2011). *C. boidinii* cells expressing VENUS under the constitutive *ACT1* promoter were inoculated onto leaves and their growth was followed by fluorescence microscopy and quantitative PCR analysis for two weeks. *C. boidinii* cells proliferated approximately 3–4 times after 11 days of inoculation. We also examined phyllosphere proliferation of gene-disrupted mutant strains impaired in methanol metabolism, peroxisome biogenesis, and autophagy. Both *aod1Δ* and *das1Δ* strains were unable to proliferate on plant leaves, suggesting that *C. boidinii* proliferation in the phyllosphere was supported by methanol assimilation on the plant leaf surface. On the other hand, gene-disrupted mutant strains lacking formaldehyde dissimilation pathway enzymes (FLD and FDH) and peroxisomal anti-oxidant enzymes (CTA and Pmp20) could proliferate. These results suggest that methylotrophic yeasts in the phyllosphere acquire energy from some other compounds that do not repress the induction of methanol-assimilation genes and that the peroxisomal anti-oxidant system was not critical for survival in the phyllosphere. On young growing leaves, the number of peroxisomes in *C. boidinii* increased in the dark period, and gene disruption of Pex5, which is responsible for peroxisomal protein import, resulted in impaired proliferation in the phyllosphere, indicating that peroxisome assembly is required for *C. boidinii* proliferation in the phyllosphere. In contrast, the numbers of peroxisomes of *C. boidinii* on young leaves decreased in the light period in which the methanol concentration was low. These findings indicate that peroxisomes were degraded by autophagy in the light period. Indeed, the *C. boidinii atg1Δ* strain (defective in Atg1, a pivotal kinase for all autophagic pathways) and *atg30Δ* strain (defective in Atg30, a pexophagy receptor) were unable to proliferate on plant leaves. Thus, peroxisome biogenesis and degradation (pexophagy, a selective autophagy for peroxisome degradation) were dynamically regulated in the phyllosphere.

We also investigated nitrogen utilization of *C. boidinii* in the phyllosphere (Shiraishi *et al*., 2015). *C. boidinii* can utilize several nitrogen sources including ammonium, nitrate, and methylamine. We tested whether the metabolism of nitrate or methylamine was required for growth and survival on plant leaves by analyses of gene expression and gene disruption of nitrate reductase (YNR) or amine oxidase (AMO). On young growing leaves of *A. thaliana*, the *YNR1* gene, not the *AMO* gene, was expressed, and its expression level fluctuated during the daily cycle. Since the *C. boidinii ynr1Δ* strain was unable to proliferate on young leaves, nitrate was concluded to be the major nitrogen source for *C. boidinii* on growing plant leaves.

Expression of the *AMO1* gene was not observed on young leaves, but the *AMO1* gene was significantly expressed in cells inoculated on wilting leaves. These results suggest that *C. boidinii* utilizes nitrate on young leaves, whereas the available nitrogen source changed and the yeast started to assimilate methylamine as a nitrogen source on aged leaves. Furthermore, *C. boidinii* YNR, which was essential for proliferation on young leaves, was transported to and degraded in the vacuole on aged leaves, by the cytoplasm-to-vacuole targeting (Cvt) pathway, one of the selective autophagic pathways.

In contrast to young leaves, the methanol concentration on wilting or dead leaves was estimated to be greater than 250 mM and did not show diurnal oscillation (Kawaguchi *et al.*, 2011). Huge developed peroxisomes were observed in *C. boidinii* inoculated onto these leaves. Since AMO is also a peroxisomal enzyme, peroxisomes of methylotrophic yeasts on the aged plant leaves may play important roles both in carbon and nitrogen metabolism. Peroxisomes are thought to be storage organelles for proteins that served as a source of amino acids in the natural environment because non-motile and asporogenous yeast cells on dead plants must survive until they obtain nutrients for further proliferation (Fig. 11.2).

Thus, methylotrophic yeasts proliferate and survive on plant leaf surfaces by adapting to environmental nutrient sources, which change not only during the day-night cycle but also during the life cycle of the plant. Such metabolic regulation was demonstrated not only by gene expression but also by autophagy (Fig. 11.2).

Transcription factors involved in regulation of methanol-inducible gene expression

When cells pre-grown on carbon sources other than methanol are transferred to methanol medium, cells must adapt to methanol medium by induction

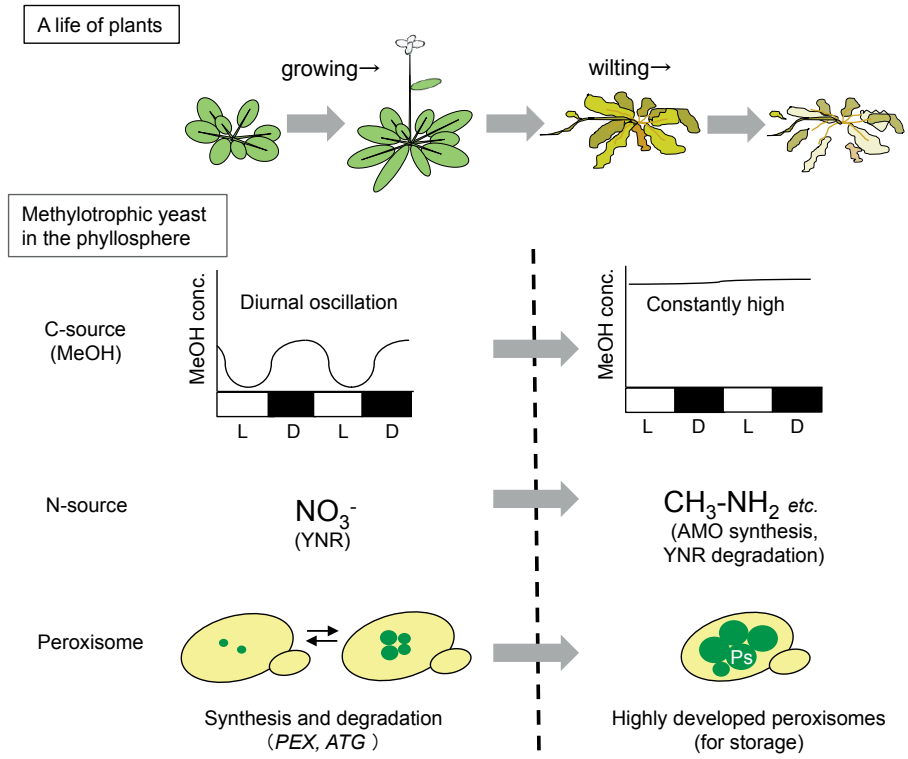

Figure 11.2 Lifestyle of methylotrophic yeasts on plant leaves. Methanol assimilatory enzymes and nitrate reductase (YNR) were required for proliferation of *C. boidinii* on young growing leaves. The methanol concentration (MeOH conc.) showed diurnal oscillation: high in the dark period (D) and low in the light period (L). Responding to methanol in the dark period, cells induced the methanol-inducible genes and peroxisomes (Ps). Responding to depletion of methanol in the light period, peroxisomes were degraded by autophagy. Not only synthesis of peroxisomes (conducted by *PEX* gene products), but also their autophagic degradation (conducted by *ATG* gene products) were necessary for *C. boidinii* to proliferate on young leaves.
On wilting or dead leaves, the MeOH conc. is generally high and peroxisomes are massively developed. Peroxisomes may function as storage organelles for proteins as a source of amino acids. While nitrate is the major nitrogen sources on young leaves, YNR is degraded by a type of selective autophagic pathway, the Cvt pathway, on wilting leaves. Under these conditions, AMO is induced to utilize methylamine as a nitrogen source.

of methanol metabolic genes and peroxisome proliferation. Since this metabolic shift includes drastic intracellular reorganization in addition to methanol-induced gene expression, it takes some time, ca. 2 h, before methanol consumption begins. During the metabolic shift, cells in transition were revealed to be a heterogenous population that exhibited differing levels of the methanol-induced gene expression based on single-cell analysis (Takeya *et al.*, 2018).

Based on the expression levels of methanol-inducible genes in media containing different carbon sources, there are three different regulatory states for methanol-inducible genes (Fig. 11.3) (Yurimoto *et al.*, 2000, 2011; Yurimoto, 2009). Glucose or ethanol completely represses methanol-inducible gene expression even in the presence of methanol [(i) repression]. Glycerol also represses methanol-inducible genes in *P. pastoris,* but not in *C. boidinii* and *H. polymorpha*. After depletion of the carbon source during metabolic shift (glucose in Fig. 11.3), repression is released and the genes are activated by (ii) derepression (independent of the presence of methanol). Finally, the presence of methanol induces maximum expression of methanol-inducible genes via (iii) methanol induction (Fig. 11.3) (Hartner *et al.*, 2006; Yurimoto *et al.*, 2011). So far, several transcription factors involved in regulation of methanol-inducible genes have been identified and characterized in *C. boidinii*, *H. polymorpha*, and *P. pastoris*. Gene disruption analyses revealed that each transcription factor is involved in one of the steps of gene regulation. Therefore, full activation of methanol-inducible genes is achieved by these three events in this order, (i) repression, (ii) derepression, and (iii) methanol induction, all of which are mediated by different transcription factors.

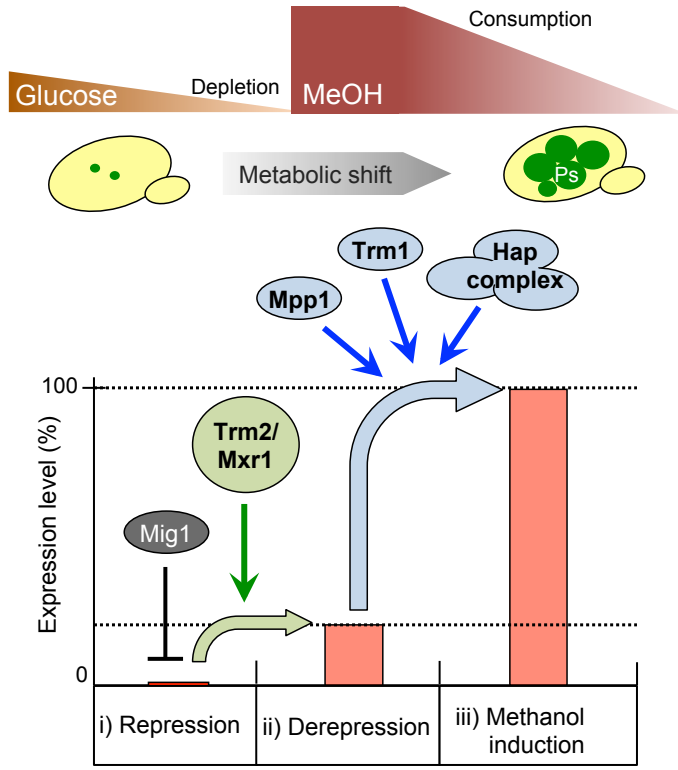

Figure 11.3 Regulation of methanol-inducible genes by repression and two-stepwise activation with transcription factors. Cells pre-grown on glucose adapt to the new carbon source methanol (MeOH) by induction of genes encoding metabolic enzymes and peroxisome (Ps) proliferation. Expression of methanol-inducible genes can be dissected into the following three regulatory events: (i) repression; (ii) derepression; and (iii) methanol induction. When glucose-grown cells are transferred to methanol medium, at first, (i) repression (regulated by Mig1) is released with consumption of glucose, followed by stepwise gene activation via (ii) derepression (by CbTrm2 and PpMxr1) and (iii) methanol-induction (by Mpp1, Trm1, and the Hap complex).

In *S. cerevisiae*, the key transcription factor of glucose repression is ScMig1, a zinc finger protein that binds to promoters of glucose-repressible genes (Klein *et al.*, 1998; Carlson, 1999; Schüller, 2003; Turcotte *et al.*, 2010). Homologues of ScMig1 in *H. polymorpha* and *C. boidinii* have been identified and characterized (Stasyk *et al.*, 2007; Zhai *et al.*, 2012). Analyses of the transcript level of the methanol-inducible *C. boidinii AOD1* gene in the *Cbmig1Δ* strain revealed that CbMig1 represses methanol-inducible genes in glucose-containing medium (Zhai *et al.*, 2012).

With respect to the transcription factors involved in (ii) derepression, homologues of the C_2H_2-type transcriptional factor ScAdr1, i.e. PpMxr1 and CbTrm2 were identified (Lin-Cereghino *et al.*, 2006; Sasano *et al.*, 2010). ScAdr1 functions in the activation of various genes involved in the metabolism of non-fermentable carbon sources such as glycerol, ethanol and oleate, as well as in peroxisome biogenesis (Simon *et al.*, 1991; Turcotte *et al.*, 2010). PpMxr1 and CbTrm2 share a number of functional similarities in the regulation of methanol-inducible gene expression, including binding to methanol-inducible promoters and subcellular localization (Lin-Cereghino *et al.*, 2006; Sasano *et al.*, 2010). However, growth of the gene-disrupted strains on non-fermentable carbon sources was different between *P. pastoris* and *C. boidinii*. The *Cbtrm2Δ* strain was unable to grow on methanol and oleate, and showed a reduced rate of growth on glycerol, but this strain did not exhibit growth defect on glucose and ethanol. On the other hand, the *Ppmxr1Δ* strain exhibited growth defects on methanol, ethanol, oleate, and glycerol, but no growth defect on glucose. CbTrm2 may have additional regulatory functions during ethanol metabolism since CbTrm2 is localized to the nucleus during growth on ethanol. PpMxr1 was shown to be inactivated by its phosphorylation in ethanol-grown cells (Parua *et al.*, 2012).

Several transcription factors unique to methanol induction are thought to be specific to and conserved only in methylotrophic yeasts. Three such transcription factors have been identified and characterized as responsible for (iii) methanol induction, i.e. (i) Mpp1 (HpMpp1 and PpMit1) (Leao-Helder *et al.*, 2003; Wang *et al.*, 2016), (ii) Trm1 (CbTrm1 and PpPrm1) (Sasano *et al.*, 2008; Sahu *et al.*, 2014), and (iii) the CbHap complex (Oda *et al.*, 2015; Oda *et al.*, 2016). Disruption of these genes resulted in severe growth defects on methanol but not on glucose or other non-fermentable carbon sources such as ethanol and glycerol. Mpp1 and Trm1 proteins belong to the $Zn(II)_2Cys_6$-type zinc cluster protein family. Mpp1 proteins were shown to be induced by methanol, whereas Trm1 proteins were constitutively expressed (Sasano *et al.*, 2008; van Zutphen *et al.*, 2010). Analysis of a double mutant lacking CbTrm1 and CbTrm2 and analyses of PpMit1 expression in gene disruptants of *PpMXR1* or *PpPRM1* revealed that Trm2 or Mxr1-dependent derepression occurred before methanol induction, which requires Trm1-dependent expression of Mpp1 (Fig. 11.3) (Sasano *et al.*, 2010; Wang *et al.*, 2016).

Recently, we revealed that the CbHap complex functions in (iii) methanol induction (Oda *et al.*, 2015, 2016). The Hap complex, a stable heterotrimer (Hap2/3/5), is highly conserved among all eukaryotes, from yeasts to humans and binds to a CCAAT consensus sequence (Buschlen *et al.*, 2003; McNabb *et al.*, 2005). In *S. cerevisiae*, the Hap complex together with another component, Hap4, activates genes involved in respiratory metabolism and mitochondrial biogenesis. Although the Hap complex functions in the metabolism of various non-fermentable carbon sources in methylotrophic yeasts, the CbHap complex was specifically responsible for methanol induction, since *Cbhap2Δ*, *Cbhap3Δ*, and *Cbhap5Δ* showed decreased transcript levels of methanol-inducible genes, resulting in a severe growth defect on methanol but not on glucose and other non-fermentable carbon sources (Oda *et al.*, 2015). Comparison of amino acid sequences of CbHap3 and ScHap3 revealed that CbHap3 contains a highly conserved region including a DNA binding motif in the N-terminal half of the protein. In addition, CbHap3 has a unique extended amino acids sequences at its C-terminus, which is conserved among methylotrophic yeasts but not present in ScHap3. Deletion of this C-terminal region of CbHap3 resulted in a decrease in the transcript level of methanol-induced genes but did not affect DNA-binding activity (Oda *et al.*, 2016). Furthermore, expression of CbHap3 in the *Schap3Δ* strain could complement the growth defect on glycerol, although expression of ScHap3 in the *Cbhap3Δ* strain could not complement the growth defect on methanol. Based on these results,

it was concluded that the C-terminal extended region plays a unique role in (iii) methanol induction and the N-terminal region of CbHap3 has conserved roles, such as DNA binding and interaction with Hap2 and Hap5. We propose that, during evolution, Hap3 proteins of methylotrophic yeasts may have acquired the specific function of activating the methanol-inducible genes via their unique C-terminal region.

Methanol-sensing machinery

Multiple transcription factors have been found to function in the regulation of methanol-inducible genes in methylotrophic yeasts. However, how methylotrophic yeasts sense methanol, and how the methanol-sensing signal is transmitted to transcription factors, remained open questions for a long time. In nature, methylotrophic yeasts have to adapt to environments in which the methanol concentration is constantly changing, e.g. on the surface of plant leaves. The methanol concentration on young leaves changes in the range of 0–0.2% (ca. 0–60 mM) and that on wilting leaves reaches 1% (ca. 250 mM). Methylotrophic yeasts must therefore sense a wide range of methanol concentrations in the environment and regulate the expression of methanol-inducible genes in response. We found that the transcript levels of methanol-inducible genes increased in the presence of 0.001–0.1% methanol but decreased in the presence of more than 0.1% methanol (Ohsawa *et al.*, 2017). When the methanol concentration in the environment is high, excess AOD results in the accumulation of formaldehyde, which is toxic to cells. Therefore, cells avoid formaldehyde accumulation by regulating the expression levels of the methanol-inducible genes. On the other hand, a decrease in the transcript levels of methanol-inducible genes at higher methanol concentrations (> 0.1%) prevents efficient production of heterologous proteins under the control of methanol-inducible promoters. Therefore, revealing the molecular mechanism of methanol-sensing and the methanol-signalling pathway is important not only for understanding physiological functions of methylotrophic yeasts in nature but also for efficient production of heterologous proteins from methanol.

Recently, we found that the Wsc family proteins, which are plasma membrane spanning sensor proteins conserved from yeasts to mammalian cells, function as a methanol-sensing machinery in the methylotrophic yeast *P. pastoris* (Ohsawa *et al.*, 2017). Wsc family proteins activate the cell wall integrity (CWI) signalling pathway in response to cell surface stresses (heat, osmotic shock, high concentration of ethanol). *P. pastoris* has three putative genes encoding Wsc family proteins, and PpWsc1 and PpWsc3 were revealed to be involved in regulation of methanol-inducible gene expression. Growth of the *Ppwsc1Δ* strain on methanol was severely impaired and the transcript levels of methanol-inducible genes in the *Ppwsc1Δ* strain were significantly lower than those in the wild-type strain. There were no significant differences in both the growth or in the transcript levels between the *Ppwsc3Δ* strain and the wild-type strain. But the *Ppwsc1ΔPpwsc3Δ* strain exhibited a more severe growth defect than the *Ppwsc1Δ* strain on methanol medium and the transcript levels of methanol-inducible genes in the *Ppwsc1ΔPpwsc3Δ* strain grown on methanol were notably lower at concentrations above 0.005% methanol. Thus, PpWsc1 and PpWsc3 are responsible for the methanol-dose-dependent gene expression. PpWsc1 responded to lower methanol concentrations (0.01–0.05%) than PpWsc3 (0.1–0.5%). Therefore, PpWsc1 and PpWsc3 seem to cooperatively sense a wide range of methanol for regulation of methanol-inducible genes.

The *Ppwsc1Δ* strain, but not the *Ppwsc3Δ* strain, exhibited a high-temperature sensitive phenotype. Moreover, the expression of *PpWSC1* in the *Ppwsc1ΔPpwsc3Δ* strain could complement the high temperature-sensitive phenotype, but the expression of *PpWSC3* could not. Thus, PpWsc1 functions both in the high temperature stress response and in methanol-induced gene expression, while PpWsc3 functions specifically in methanol-induced gene expression. The *Ppwsc3Δ* strain grew normally on methanol at 28˚C, but exhibited a severe growth defect on methanol at 37°C. During growth on methanol at high temperature, PpWsc1, which has to respond to both high-temperature stress and methanol, is likely to be more involved in the high-temperature stress response, and PpWsc3 may make a greater contribution to methanol sensing (Fig. 11.4).

To investigate whether PpWsc1 distinguishes two different environmental stimuli, i.e. high

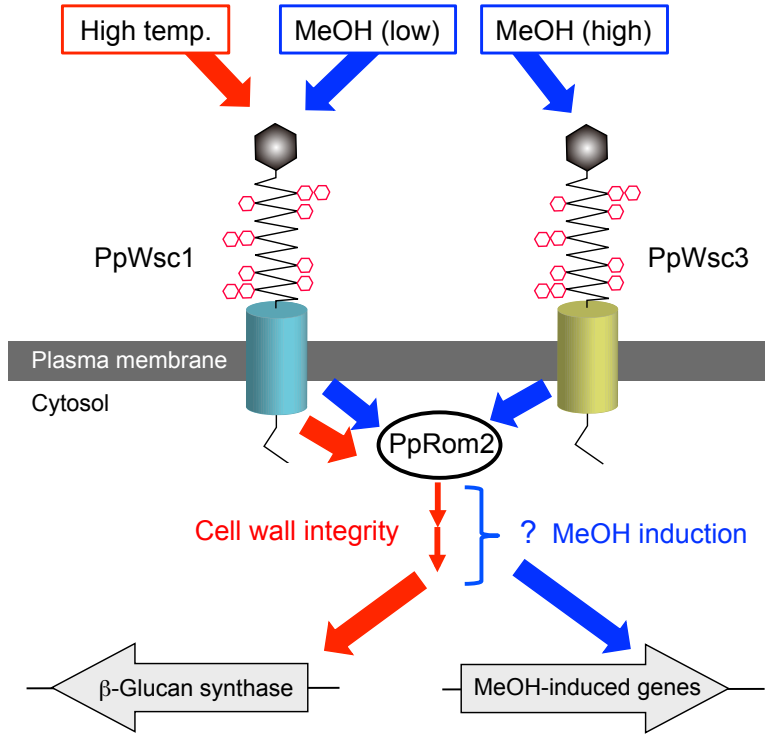

Figure 11.4 Proposed model of *P. pastoris* Wsc family protein function in methanol induction. PpWsc1 and PpWsc3 function in methanol sensing, for lower [MeOH (low)] and higher methanol concentrations [MeOH (high)], respectively. PpWsc1 also functions in the high temperature (High temp.) stress response, activating heat stress-inducible genes such as β-glucan synthase via the cell wall integrity (CWI) pathway. Both PpWsc1 and PpWsc3 interact with PpRom2 to activate methanol-inducible genes. We speculate that the methanol-sensing signal branches off from some point in the CWI pathway. The signal transduction pathway for the high temperature stress response is indicated by red arrows and that for methanol sensing is indicated by blue arrows.

temperature and methanol, we screened for site-directed mutants of PpWsc1 that were impaired in either methanol-induced gene expression or the high temperature stress response. A PpWsc1(Y53A) alanine substitution mutant was found to have decreased transcript levels of methanol-inducible genes compared with the wild-type PpWsc1, but did not show a high-temperature sensitive phenotype. On the other hand, a PpWsc1(Y53F) phenylalanine substitution mutant became sensitive to high temperature stress, but retained the normal transcript level of methanol-inducible genes. These results indicate that PpWsc1 senses both high temperature and methanol by different mechanisms. We hypothesize that PpWsc1 may adopt two different configurations in response to high temperature stress and methanol-sensing.

Rom2, interacting with Wsc family proteins, was known to be a downstream component in the CWI signalling pathway of *S. cerevisiae*. Interaction of PpWsc1 with PpRom2 was revealed to be responsible for both methanol induction and the CWI pathway (Fig. 11.4). Further investigation is necessary to elucidate the involvement of other components of the CWI pathway in the methanol induction pathway.

Development of a single-cell biosensor for detection of enzymatically produced methanol

Methanol is a promising alternative carbon source to replace coal and petroleum (Olah, 2005; Olah *et al.*, 2006), and efficient processes for methanol

production from methane or renewable biomass are expected to be established. Methanol-producing enzymes, such as pectin methylesterase (PME) and methane monooxygenase, are candidates for enzymatic production of methanol. To exploit methanol-producing enzymes with high activity, a sensitive and high-throughput method to evaluate their activities at the single-cell level should be established. Methanol biosensors have been developed with microbial cells or immobilized enzymes; however, these biosensors are not appropriate for the detection of methanol that is endogenously produced by recombinant enzymes (Guilkbault *et al*., 1983; Wen *et al*., 2014). The detection limit of the *C. boidinii* methanol sensor, which is based on expression of the fluorescent protein gene under the control of the methanol-inducible *CbDAS1* promoter, was 0.25 mM (Kawaguchi *et al*., 2011). However, a more sensitive sensor would be required to evaluate enzymatically produced levels of methanol by high-throughput analysis such as fluorescence-activated cell sorting (FACS).

Recently, we developed a *P. pastoris* methanol sensor and optimized the culture and methanol assay conditions by FACS (Takeya *et al*., 2018). We successfully improved the detection limit to 2.5 μM, which is 100-fold lower than that of the fluorescence microscopy-based *C. boidinii* methanol sensor. Interestingly, *aox1Δ* cells, which are impaired in methanol metabolism, exhibited lower cellular fluorescence intensity than the wild-type cells, suggesting that the methanol sensor cells need to metabolize methanol for maximum production of the fluorescent protein. The preculture conditions of the sensor cells were also found to be critical for the sensitive detection of methanol. During preculture on glucose medium, methanol-inducible gene expression is completely repressed by glucose until mid-exponential phase, but glucose repression is released in late-exponential phase cells. Therefore, the physiological state of late-exponential phase cells is suitable for a sensitive response to low concentrations of methanol.

To substantiate the feasibility of this methanol biosensor for evaluating the activity of methanol-producing enzymes, the *Aspergillus niger* PME gene was heterologously expressed in the *P. pastoris* methanol sensor cells, and we revealed that the level of cellular enzyme activity correlated with the level of cellular fluorescence intensity. Thus, this methanol biosensor can be used for high-throughput screening of single cells harbouring an enzyme with high methanol-producing activity.

Conclusions

Methylotrophic yeasts have been used as hosts for heterologous protein production and model organisms to study peroxisome homeostasis for a long time, and understanding the transcription factors for methanol-inducible gene expression and peroxisome dynamics has been progressing. However, so far, we did not understand why methylotrophic yeasts have come to harbour such physiological traits as strong methanol-inducibility of gene expression and dynamic regulation of peroxisomal development and degradation, and how they live in nature. Regarding this point, we found that methylotrophic yeasts can proliferate on plant leaves where concentrations of methanol oscillate with the daily light–dark cycle (Kawaguchi *et al*., 2011). Simultaneously, we established analytical methods that measure cell proliferation, gene expression, and intracellular organelle dynamics, and we elucidated metabolic and physiological functions required for the phyllospheric life of methylotrophic yeasts at the molecular level. We also constructed methanol sensor cells using methylotrophic yeasts expressing a fluorescent protein under the control of methanol-inducible gene promoters (Takeya *et al*., 2018). These sensor cells were used to determine the methanol concentrations on plant leaves and the activity of methanol-producing enzymes.

In contrast to the transcription factors involved in methanol-inducible gene expression, the methanol-sensing machinery and associated signal transduction pathway have not been investigated in detail. Wsc family proteins, which are known to be cell-surface stress sensor proteins, are involved in methanol-sensing machinery in methylotrophic yeasts (Ohsawa *et al*., 2017). PpWsc1 has two distinct functions, methanol sensing and response to high-temperature stress, and PpWsc3 specifically functions in methanol sensing. As in the case with the C-terminal conserved region of Hap3 in methylotrophic yeasts (Oda *et al*., 2016), well-conserved Wsc family proteins have evolved to acquire methanol-sensing function in these yeasts.

Future trends

In this chapter, we described the recently obtained information about the physiological functions of methylotrophic yeasts in the phyllosphere. Our methylotrophic yeast-plant system, which includes newly established analytical methods, can combine molecular cellular biology with ecology, and can be used to explore the yeast life cycle at the molecular level in nature. Further studies on yeast methylotrophy in the phyllosphere will facilitate the characterization of new physiological functions and their importance in natural environments. A detailed understanding of the molecular basis of yeast methylotrophy, including gene activation, methanol-sensing machinery, and signal transduction pathways, will also lead to the development of new technology and applications, e.g. efficient heterologous gene expression and enzyme engineering by methanol-sensing technology.

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