Mannosylerythritol lipids secreted by phyllosphere yeast *Pseudozyma antarctica* is associated with its filamentous growth and propagation on plant surfaces

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Abstract The biological function of mannosylerythritol lipids (MELs) towards their producer, *Pseudozyma antarctica*, on plant surfaces was investigated. MEL-producing wild-type strain and its MEL production-defective mutant strain (*ΔPaEMT1*) were compared in terms of their phenotypic traits on the surface of plastic plates, onion peels, and fresh leaves of rice and wheat. While wild-type cells adhering on plastic surfaces and onion peels changed morphologically from single cells to elongated ones for a short period of about 4 h and 1 day, respectively, *ΔPaEMT1* cells did not. Microscopic observation of both strains grown on plant leaf surfaces verified that the wild type colonized a significantly bigger area than that of *ΔPaEMT1*. However, when MELs were exogenously added to the mutant cells on plant surfaces, their colonized area became enlarged. High-performance liquid chromatography analysis revealed a secretion of higher amount of MELs in the cell suspension incubated with wheat leaf cuttings compared to that in the suspension without cuttings. Transcriptional analysis by real-time reverse transcriptase PCR verified that the expression of erythritol/mannose transferase gene and MELs transporter gene of *P. antarctica* increased in the cells inoculated onto wheat leaves at 4, 6, and 8 days of incubation, indicating a potential of *P. antarctica* to produce MELs on the leaves. These findings demonstrate that MELs produced by *P. antarctica* on plant surfaces could be expected to play a significant role in fungal morphological development and propagation on plant surfaces.

Keywords Mannosylerythritol lipids · *Pseudozyma antarctica* · Plant surface · Propagation · Colonization

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Introduction

Basidiomycete yeasts of the genus *Pseudozyma* (*Ustilaginales*) generally inhabit various phyllospheres (Allen et al. 2004; Boekhout 2011; Kitamoto et al. 2011; Morita et al. 2012; Seo et al. 2007). Several species of this genus have been reported to possess beneficial function for plants, such as biological control (Avis and Bélanger 2002; Cheng et al. 2003; Jarvis et al. 1989), inducing resistance against plant pathogens (Buxdorf et al. 2013). In addition, this genus is known to have intrinsic properties which include the ability to produce various biotechnological materials, such as α-amylase and lipase-B (Boekhout 2011), as well as biosurfactants (Amaral et al. 2010; Banat et al. 2010; Kitamoto et al. 2002). Glycolipid biosurfactants such as mannosylerythritol lipids (MELs) that contain 4-*O*-β-D-mannopyranosyl-*meso*-erythritol as hydrophilic moiety and acetyl groups and fatty acids as hydrophobic moiety are abundantly produced by *Pseudozyma antarctica* in liquid culture particularly when vegetable oils are used as carbon source (Kitamoto et al. 2002; Morita et al. 2007). MELs are known to have potential applications in the manufacture and/or use of various industrial and medical materials. They act as anti-agglomeration agents for ice-slurry (Kitamoto et al. 2001b) and as ingredient for skin care products (Yamamoto et al. 2012) because of their superior interfacial properties (Kitamoto et al. 2002). However, their biological and

ecological roles towards their fungal producers in natural environment are still unclear.

Although the precise functions of biosurfactants are still under investigation, several insights into the activities of biosurfactants produced by *Pseudozyma* spp. and a closely related genus, *Ustilago* (Boekhout 2011), which led to some ecological impacts against its own and other fungal behaviors, have already been reported. Flocculosin, a cellobiose lipid produced by *Pseudozyma flocculosa*, has been known to show antimicrobial activity against powdery mildew fungi and Gram-positive bacteria (Hammami et al. 2011; Mimée et al. 2009). Biosurfactants of *Ustilago maydis* were reported to provide their producers an advantage to inhabit natural environments. Its ustilagic acid was reported to have an antifungal activity (Teichmann et al. 2007), contributing to the predominance of fungal colonization by eliminating other competitive microorganisms. Moreover, its cellobiose lipid was found to have a possible hormonal role in promoting mating-type recognition (Hewald et al. 2005). It may also have a strategic function in the adaptation of the fungus to its surrounding environment through enhancement of its mating efficiency.

However, even though high MELs productivity of *P. antarctica* in liquid culture has already been shown, their productivity on plant surfaces remains unclear, and if ever they are produced, the nutrients necessary for MELs production are still unknown. Since the presence of *P. antarctica* is recognized to be widespread on various plants (Boekhout 2011), understanding the functional role of MELs both for their producers and for other microorganisms inhabiting plants could be expected to give light to the biological function of phyllosphere microorganisms in the environment. Culture conditions and biosynthetic pathway, as well as the genes responsible (Morita et al. 2010) for MELs production have already been well elucidated (Kitamoto et al. 2002). With these knowledge, we aimed to determine the functional role of MELs for *P. antarctica* inhabiting plant surfaces.

The first objective of this study was to determine the specific role of MELs in the growth of *P. antarctica* on plant surfaces. Emphasis was placed on the effect of these compounds on the morphological changes in this fungus following its colonization of plant surfaces. Based on a recent report highlighting the ability of MELs to induce morphological changes in *P. antarctica* cells in liquid culture (Morita et al. 2010), it could be inferred that they can also cause similar morphological changes in *P. antarctica* cells on solid surfaces. To verify this inference, *P. antarctica* strain T-34 and its genetically modified strain defective in MELs production (Morita et al. 2010) were investigated in this study. We compared their phenotypic traits on modified solid surfaces and fresh plant surfaces. MELs that were found to promote filamentous cell growth on hydrophobic surfaces of model substrates, such as plastic films and onion peels, were examined for their effect on the propagation of fungal growth on leaf

surfaces of rice and wheat; these crops, which belong to the Gramineae family, are recognized to harbor well this fungal species (Allen et al. 2004; Kitamoto et al. 2011; Morita et al. 2012). This study also aimed to determine the potential of *P. antarctica* for MEL production on plant leaves based on high-performance liquid chromatography analysis and transcriptional analysis by real-time reverse transcriptase PCR.

Materials and methods

Fungal strains and culture conditions

P. antarctica T-34 (named as wild type in the study) (Kitamoto et al. 1990; Kitamoto et al. 2001a; Morita et al. 2006), which has been deposited in the National Institute of Agrobiological Sciences (NIAS) Genebank, Japan (accession number, MAFF 306900), and its erythritol/mannose transferase gene (*PaEMT1*) disruptant strain (*ΔPaEMT1*), which is defective in MELs production (Morita et al. 2010), were used in this study. To prepare the seed cultures, each strain was inoculated in 300 ml Erlenmeyer flask containing 30 ml FMM medium (0.2 % $NaNO_3$, 0.02 % KH_2PO_4, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.1 % yeast extract) supplied with 4 % glucose as carbon source, and shaken (200 rpm) in a rotary shaker for 1 or 4 days at 25 °C in the dark. To prepare the cell suspension, the wet cells obtained from seed cultures by centrifugation ($1,700 \times g$, 5 min) were washed twice with water and were suspended in water to the desired cell concentration.

Preparation of MELs solution

According to published reports, MELs, which is a mixture of MEL-A, MEL-B, and MEL-C (Fig. 1a), were produced from soybean oil (Wako, Tokyo, Japan) by the wild-type *P. antarctica* (Kitamoto et al. 1990; Morita et al. 2010). Crude MELs were extracted with equal volume of ethyl acetate and were separated by silica gel chromatography, and then the isolated MELs fraction was re-extracted with ethyl acetate (Kitamoto et al. 1990; Morita et al. 2010). The re-extracted MELs fraction was concentrated by evaporation (Morita et al. 2007). The MELs were dissolved with ethanol and was adjusted to 8 % (stock), then was further diluted to 0.04 % with water when used for experiments.

Morphological observation of *P. antarctica* cells adhering on hydrophobic plastic surface

Ten microliters aliquot of wild type or *ΔPaEMT1* cell suspension (5×10^5 cells/ml) prepared from 1-day cultures incubated as described above was applied onto hydrophobic surface of ten Gelbond film (Takara Bio Inc., Otsu, Japan) segments (approximately 1 cm×1 cm); the shape of the cells

Fig. 1 Mannosylerythritol lipids (MELs) produced by *P. antarctica*. **a** Chemical structure of MELs. **b** HPLC chromatogram of MELs in the extract obtained from the supernatant of *P. antarctica* cell suspension with leaf segments after 6 days of incubation

in five segments were immediately observed to serve as control. The remaining segments were incubated in moist chambers at 25 °C in the dark for 4 h. To observe the cells that adhered irreversibly on the plastic film surface and to remove unadhered planktonic cells, the incubated segments were immersed into 30 ml of 10 mM phosphate buffer (pH 7.0) in a 50-ml screw-capped plastic tube and were washed by vortexing with a vortex mixer (Vortex-Genie 2, Scientific Industries, New York, USA) for 30 s at maximum speed. The incubation time was selected based on the results of a preliminary experiment; the irreversible adherence (Andrews and Buck 2002) of the cells on the surfaces was confirmed to have been established after 4 h of incubation (data not shown). The washed plastic film segments were then recovered from the tube and the extra buffer solution around the segments was carefully removed using filter paper. Subsequently, lactophenol cotton blue solution was dropped onto each plastic film surface to stain the adhering cells. Stained cells were observed under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan), and the lengths of 30 randomly chosen cells from each of the five segments were measured. Differences in cell length between the strains were evaluated by Student's *t* test using KaleidaGraph 4.1 J software (Synergy Software, Reading, PA, USA). The experiment was repeated once.

Morphological observation of *P. antarctica* propagules on the surface of onion peels

To observe the differences in the fungal morphology on plant tissue surfaces attributed to MELs produced by *P. antarctica*,

peels of an onion tuber (unknown cultivar) were used as substrate. The peels were removed with tweezers and were cut with a razor blade into small segments (approximately 1 cm×1 cm), which were then fixed in 100 % ethanol for 3 days. After rinsing the peel segments with water, they were placed onto FMM agar (1.5 %) plates with the hydrophobic surface facing up. Subsequently, 2.5 μl of cell suspension (approximately 4×10^5 cells/ml) of seed cultures of wild type or *ΔPaEMT1* grown for 4 days as described above was applied onto the surface of each peel segment, and they were incubated for 24 h at 30 °C in the dark. Subsequently, the inoculated peel segments on the FMM plates were carefully transferred onto glass slides, and the surface of the peels were thoroughly rinsed by dropping 50 μl water twice, then the remaining fungal propagules on the peels were stained with lactophenol cotton blue solution as described above. The stained propagules were observed under the light microscope.

Colonization of rice and wheat leaf surfaces by *P. antarctica* propagules

Matured healthy leaves of glasshouse-grown rice plants (cv. Koshihikari, four to six leaf stages) and wheat plants (cv. Norin No.61, four to five leaf stages) were used for the experiment. The leaves were detached from the plants and cut into small segments (150–200 mm^2); then the segments were fixed onto the glass slides using a double-sided tape with their adaxial surface facing up. To mark the inoculation point, a small dot using an oil-based pen was made at the center of each leaf segment. Aliquots (5 μl) of wild type or *ΔPaEMT1* cell suspension (OD$_{600}$ was 1.0) were applied separately onto each dot, and then were air-dried in a clean bench. After 1 and 3 days of incubation in a dark moist chamber at 25 °C, the cells on the inoculated leaf surfaces were stained with 0.1 % (*w/v*) Calcofluor white (Fluorescent brightener 28) (Sigma-Aldrich, St. Louis, MO, USA) and were observed under an epifluorescent stereomicroscope (MZ 16 FA, Leica, Solms, Germany) through ultraviolet filters. Observation was done on three segments per treatment.

Effect of exogenous MELs on fungal colonization on rice and wheat leaf surfaces

To evaluate the direct effect of MELs on the morphology of *P. antarctica* grown on leaf surfaces, *ΔPaEMT1*-inoculated rice and wheat leaf segments were prepared following the above-mentioned procedure. After 1 day incubation, 1 μl of 0.04 % (*v/v*) MELs was exogenously added to each marked inoculation point in the leaf segments. Other segments similarly treated with water after inoculation as well those without any post-inoculation treatment were prepared as control. After further incubation for one more day, the fungal propagules on each leaf surface were observed by staining them with 0.1 %

Calcofluor white as described above. Three leaf segments per treatment were observed.

Measurement of growth area of *P. antarctica* propagule on leaf surface

Fluorescent microscopic images of the leaf segments treated above were taken with a digital CCD camera system (DFC 480, Leica×7.11 objective lens) and were saved as TIFF format files by using Image Manager IM50 (Leica) software. The TIFF images were binarized using Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) based on the optimized threshold value (T value) covering the histogram for each image; then the white colored area representing the fungal propagules on leaf segments in individual binary image was calculated using Image J (http://imagej.nih.gov/ij/) software. The white area was chosen by using the Wand Tool with the legacy mode of the program, and was expressed in terms of pixel units. Each calculation was made with three replicated leaf segments, and the data was subjected to ANOVA followed by Tukey's HSD tests using KaleidaGraph 4.1 J software (Hulinks Inc., Tokyo, Japan).

High-performance liquid chromatography analysis of MELs produced by *P. antarctica* from wheat leaf segments

To evaluate the potential of plant leaves to serve as inducer or nutrient sources for *P. antarctica* towards MELs production, wheat leaves were used as a representative plant material. Leaves were detached from potted plants grown in the glasshouse as described above and were cut into small segments (approximately 70–100 mm^2). The segments (3.1 g fresh weight) were floated on a petri dish (6 cm in diameter) containing 6 ml cell suspension of wild type (2×10^9 cells per ml) prepared from a 4-day-shaken seed culture as described above. Petri dishes containing only cell suspension (6 ml) were also prepared. For the control, plates containing equal volume of water with the same amount of leaf segments without cells were also prepared. After incubation for 4 h (=0 day), 2 days, 4 days, and 6 days at 30 °C in the dark, aliquots (4 ml) of cell suspension were recovered from each treated dish. The suspensions were centrifuged ($2,300\times g$, 5 min) to separate the cells from the supernatants containing MELs, which were then quantified by high-performance liquid chromatography (HPLC). MELs in each supernatant were extracted with an equivalent volume of ethyl acetate according to the procedures described above. The resulting ethyl acetate extract was dissolved with ethanol and then was adjusted to a concentration of 4 mg extract/ml. MELs in each ethanol solution were separated using a silica gel column (Inertsil SIL 100A 5 μm, 4.6×250 mm; GL Science, Tokyo, Japan) and was detected with a low-temperature evaporative light scattering detector (ELSD-LT; Shimadzu, Kyoto, Japan). The

separation was performed with a gradient solvent program consisting of various proportions of chloroform and methanol (from 100:0 to 0:100, *v/v*) at a flow rate of 1 ml/min (Rau et al. 2005). Under these conditions, MEL-A, MEL-B, and MEL-C standards were detected at retention times of approximately 6.3, 6.6, and 6.9 min, respectively (Fig. 1b), and the total area of the three peaks on the chromatogram was calculated. Calculation was performed in triplicate as technical replicates in each solution.

Expression of gene for MEL production by *P. antarctica* on wheat plant surface

Seven wheat seedlings (cv. Norin No.61, second leaf stage) grown in separate plastic seedling boxes were used for the experiment. The seedlings were spray-inoculated with 8 ml of wild-type cell suspension (7.5×10^8 cells/ml) prepared from a seed culture grown in 4 % glucose-supplied FMM as described above. After incubation for 1, 2, 4, 6, and 8 days in a glasshouse, all the inoculated parts of the seedlings in each box were detached and cut into small segments with sterilized scissors, and then immediately preserved in a freezer at −80 °C until use within 10 days. Each frozen sample (approximately 50 mg) was ground in a mortar using liquid nitrogen, and its total RNA was extracted from the ground sample using 5 ml Isogene (Nippon gene, Tokyo, Japan), then aliquots (40 μl) of the extracted RNAs dissolved in 200 μl in diethylpyrocarbonate water, of which concentrations ranged from 172.1 to 360.4 ng/μl, were treated with DNase (RQ1, Promega, Madison, WI, USA) according to manufacturer's instructions. The DNase-treated total RNAs were confirmed to be free from DNA contamination by the absence of amplicons for actin gene of the fungal strain (DDBJ/EMBL/GenBank accession number GAC77090.1), which was analyzed by PCR (a hot start of 95 °C for 2 min, followed by 28 cycles of 30 s at 95 °C , 30 s at 55 °C and 2 min at 72 °C, and 72 °C for 5 min) using the GeneAmp PCR system 9700 (Life Technologies Corporation, Carlsbad, CA, USA), with a reaction mixture containing 0.5 μM primer sets (PactinF, '5-CCGTGACATCAAGGAGAAGC-3' ; PactinR, '5-GATCGAGTTGTACGTCGTCT-3'; expected products 220 bp from cDNA), 1× PCR buffer (Takara Bio Inc.), 0.2 mM each dNTP, and 0.5 U Taq DNA polymerase (TaKaRa ExTaq, Takara Bio Inc.). Subsequently, 15 μl first strand of cDNA was synthesized from the 5 μl DNase-treated total RNA using Oligo dT primer (50 μM) and Random 6 mers (100 μM), with 0.75 μl PrimeScript RT Enzyme Mix I in PrimeScript RT reagent kit (Takara Bio Inc.) at 37 °C for 15 min followed by 85 °C for 5 min according to the manufacturer's instructions. The primers used for real-time RT-PCR were PaEMT1-609 F (GGCGACAGCGTACCTTGATG) and PaEMT1-888R (TGGATCCCGCCGACACAGTT), which were designed to amplify a partial gene of *PaEMT1* including 78-bp intron

(accession number AB548311, expected product 202 bp from cDNA) encoding an erythritol/mannose transferase of *P. antarctica* (Morita et al. 2010), and PaMMF1-3 F ('5-GGACGACAAGATCGCGCTGA-3') and PaMMF1-249R ('5-AGTTCGCCGCGATCGACGAA-3'), which were designed to amplify a partial gene of *PaMMF1* including 59-bp intron (accession number DF196785-5, expected product 196 bp from cDNA), a putative MEL transporter gene (*mmf1*) of *U. maydis* (Hewald et al. 2006) based on the expressed sequence tags of *P. antarctica* (Morita et al. 2006). As previously reported, actin gene was also amplified as an internal control using the above-mentioned primers, PactinF and PactinR (Morita et al. 2006). To generate standard curves for quantifying the RT amplicons of each gene, 4–5 points of 10-fold serial dilutions of amplicons from cDNA of each gene, which were obtained by PCR using the above-mentioned reaction conditions and individual primer set, were prepared as templates. We followed the method of the real-time RT-PCR with each reaction mixture (10 μl) containing 1 μl cDNA, 5 μl SYBR Premix Ex Taq II (Takara Bio Inc.), 0.2 μl each primer (10 μM), 0.2 μl ROX Reference Dye, and 3.4 μl ddH2O, and subjected it to amplification using Applied Biosystems StepOnePlus (Life Technologies Corporation) as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and dissociation stage (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s). For the negative control, reaction mixtures without cDNA were also amplified. RT-PCR analysis for each cDNA sample was carried out in duplicate wells and replicated three times. Data was analyzed using the StepOne Software v2.1 (Life Technologies Corporation) in which C_t value was set automatically. The quantity of amplicons, representing the expression level of *PaEMT1* and *PaMMF1,* was normalized based on that of actin gene (Morita et al. 2006) in each cDNA sample, and the relative level of expression of these genes in the samples was calculated. The above experiment (trial 1) was repeated, except for the preparation of cDNA at 2 days after inoculation (trial 2). The relative gene expression levels in the two trials were combined, and subjected to Wilcoxon signed-rank test using KaleidaGraph 4.1 J software.

Results

Morphological observation of *P. antarctica* cells adhering on hydrophobic plastic surface

Cells of two *P. antarctica* strains, wild type and *ΔPaEMT1,* showed different morphologies on hydrophobic plastic surfaces after 4 h of incubation (Fig. 2). Although cells of both strains initially formed typical yeast-like cells (Fig. 2a, b), only those of the wild type immediately became elongated. The elongation was observed on either one or both ends of the

cells which then developed filamentous-growth forms (Fig. 2c). The average and standard error (SE) of the length of the filamentous wild-type cells was 21.7±0.9 μm after 4 h of incubation. On the other hand, *ΔPaEMT1* cells have relatively limited elongation (Fig. 2d) and were significantly ($P<0.01$) shorter (10.3±0.7 μm) than those of wild type. Similar differences in cell length between the two strains were observed when the experiment was repeated.

Morphological observation of *P. antarctica* propagules on the surface of onion peels

Figure 3 shows the cell morphologies of wild type and *ΔPaEMT1* on onion peel surfaces after 24 h of incubation. Most seed culture cells of both strains before inoculation were found to form similar yeast-like shape (Fig. 3a, b). The yeast-like cells incubated for a day showed that most cells of wild-type strain grew and developed long hyphae (Fig. 3c), resembling the characteristics of those on the Gelbond film (Fig. 2c). In contrast, strain *ΔPaEMT1* cells exhibited only a little change in morphology (Fig. 3d). Although some mutant strain cells became elongated and formed pseudohypae-like cells, their lengths were obviously shorter than those of the wild-type hyphae.

Colonization of rice and wheat leaf surface by *P. antarctica* propagules

After 1 day of incubation on rice leaves, propagules of both strains became localized at the inoculation sites (Fig. 4a, b), and the average sizes of their colonized areas were not significantly different from each other (Fig. 4c). However, the colonized areas after 3 days of incubation differed significantly ($P<0.05$), with *ΔPaEMT1* propagules showing restricted colonization whose area was over sixfold smaller than that of the wild type (Fig. 4b, c). Similarly, differences in the sizes of colonized areas on wheat leaves were observed between the strains; the colonized area by *ΔPaEMT1* propagules was significantly ($P<0.05$) smaller than that of wild type after 3 days of incubation, although the area at 1 day after incubation did not significantly differ from each other (Fig. 4c).

Effect of exogenous MELs on *P. antarctica* colonization on rice and wheat leaf surfaces

Figure 5 shows the microscopic images (a) and pixel-based sizes of propagules (b) of *ΔPaEMT1* with and without post-inoculation treatment with water or MELs on rice and wheat leaves. On rice leaves, colonization by the fungal propagules was seen as fluoresced area localized in the spot where the cell suspension was added (Fig. 5a). Similarly, restricted colonization by the propagules was observed on the leaf segments added with water after 1 day of incubation. On the other hand,

Fig. 2 Cells of *P. antarctica* wild type (**a**, **c**) and *ΔPaEMT1* (**b**, **d**) on hydrophobic plastic surfaces after 0 (**a**, **b**) and 4 h (**c**, **d**) of incubation. Cells were stained with lactophenol cotton blue solution. *Bars* = 20 μm
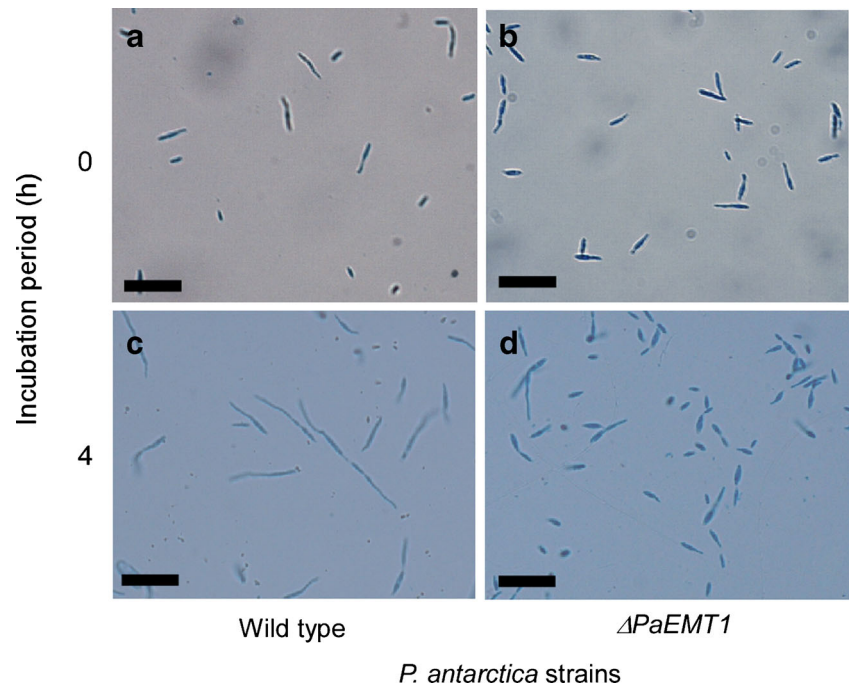
the area colonized by *ΔPaEMT1* became more developed by the post-inoculation addition of 0.04 % MELs to the inoculation site. At the edges of the MELs-supplied propagules, the cells developed into filamentous forms, which were not observed in the propagules on the segments in the control treatments (Fig. 5a). Based on the pixels of fluoresced area, the areas colonized by the fungal propagules on the MELs-treated

leaf segments were significantly (*P*<0.05) larger than those on other treated segments (Fig. 5b). On wheat leaf segments, similar enhanced colonization by the propagules was observed with post-inoculation treatment with MELs compared to those in the control treatments (Fig. 5a); the calculated areas of the MELs-treated propagules on the segments were also significantly (*P*<0.05) larger than those in the control treatments (Fig. 5b).

Fig. 3 Cells of *P. antarctica* wild type (**a**, **c**) and *ΔPaEMT1* (**b**, **d**) before inoculation (**a**, **b**) and 24 h after inoculation to hydrophobic onion peels (**c**, **d**). Cells were stained with lactophenol cotton blue solution. *Bars* = 10 μm
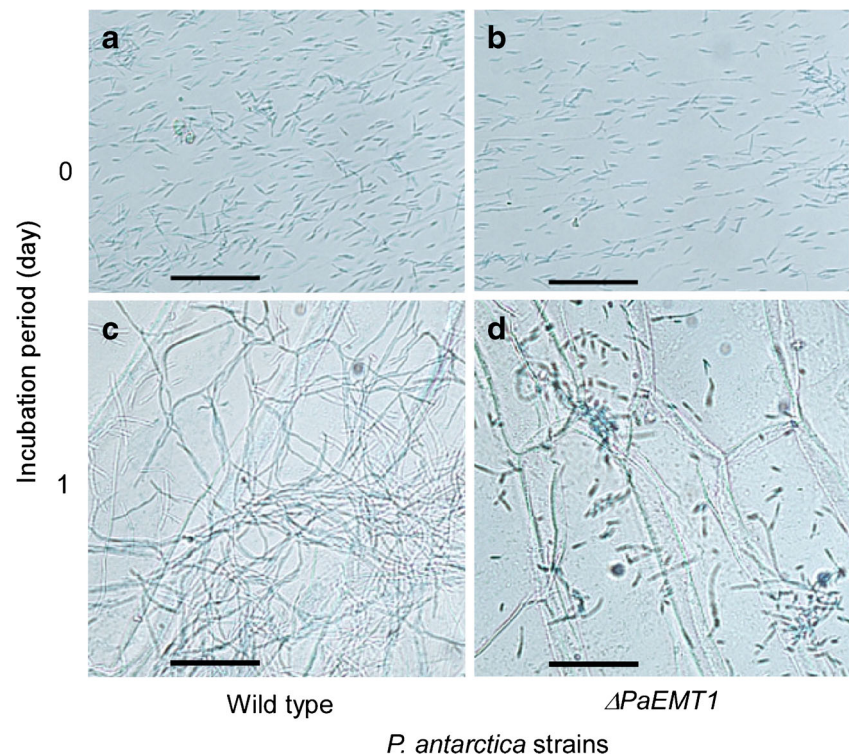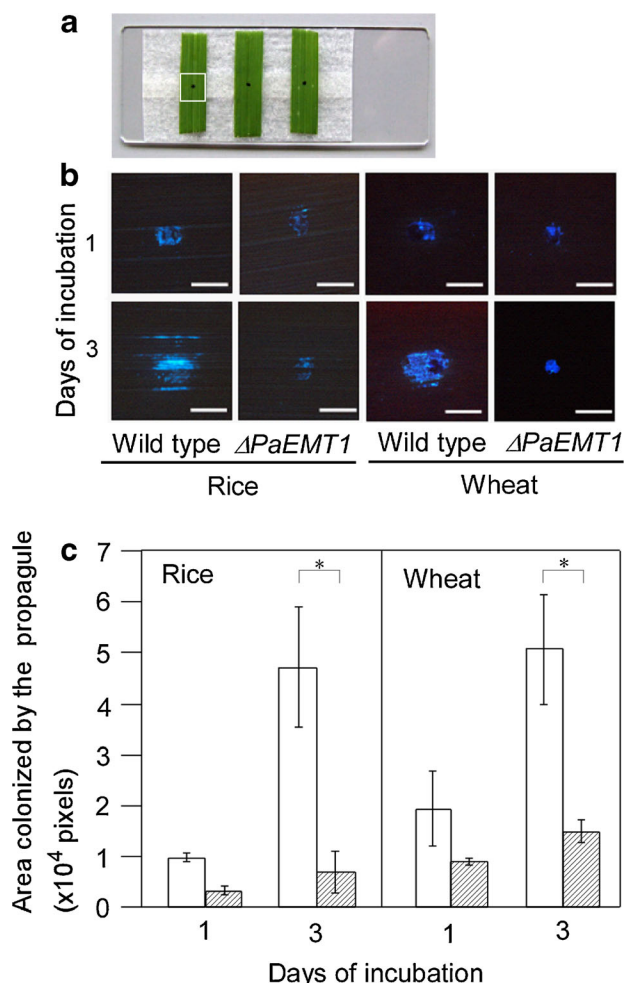
Fig. 4 Images and area of colonization by *P. antarctica* wild type and *ΔPaEMT1* on rice and wheat leaf surfaces. **a** Cell suspension of each strain was placed onto a designated inoculation site at the center of each leaf segment marked with a *black dot*. Area in a designated square on each leaf segment was observed using epifluorescent stereomicroscopy. **b** Representative fungal propagules viewed on each leaf segment after 1 and 3 days of incubation. Propagules colonizing the surfaces were recognized as *blue-white* fluorescence by staining them with 0.1 % Calcofluor white. *Bars* = 1 mm. **c** The area on each leaf segment colonized by the fungal strains. *P. antarctica* strains: *white column*, wild-type; *oblique-lined column*, *ΔPaEMT1*. Calculation was performed based on fluorescent area converted to pixel units using Image J software. *Each value* represents the average of three determinations and each *error bar* indicates the standard error of the mean. *Asterisk* means the values were significantly different (*P*<0.05) according to Student's *t* test

High-performance liquid chromatography of MELs produced by *P. antarctica* from wheat leaf segments

The MELs present in ethyl acetate extracts obtained from each cell suspension with and without wheat leaves were quantified by HPLC. Figure 6 shows the relative amount of MELs in each extract calculated based on the peak area of the chromatograms; the respective amount of MELs present in the extract from each cell suspension with and without the leaf segments after only 4 h of incubation (0 day) was regarded as
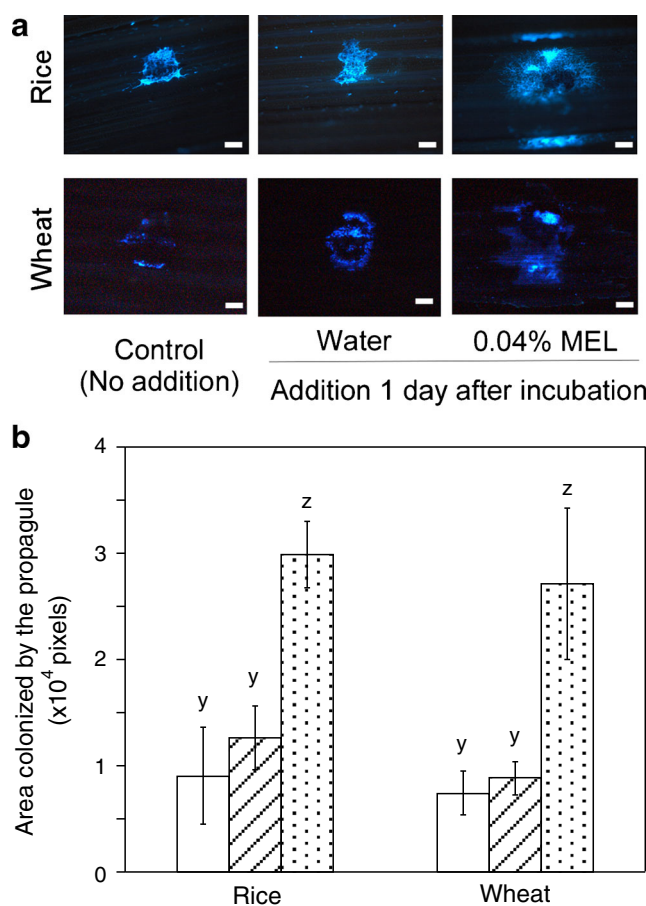
Fig. 5 Images and area colonized by *P. antarctica* *ΔPaEMT1* with or without MELs treatment after inoculation on rice and wheat leaf surfaces. **a** Representative fungal propagules on each leaf segment viewed with an epifluorescent microscope after 2 days of incubation. Propagules colonizing the surfaces were recognized as blue-white fluorescence by staining with 0.1 % Calcofluor white. *Bars* = 200 μm. **b** The area on each leaf segment colonized by the fungal strains post-treated with or without MELs. Treatment: *white column*, without post-treatment; *oblique-lined column*, post-treated with water; *dotted column*, post-treated with 0.04 % MELs. Calculation was performed based on fluorescent area converted to pixel units using the software Image J. *Each value* represents the average of three determinations and each *error bar* indicates the standard error of the mean. Different letters (*y, z*) on columns of each plant leaf are significantly different (*P*<0.05) according to ANOVA followed by Tukey's HSD tests

1.0. The relative amounts of MELs in the extracts from each cell suspension without leaf segments changed only slightly during incubation, whereas those in the extracts from each cell suspension with leaf segments increased by 2.5, 9.7, and 3.4 times after 2, 4, and 6 days of incubation, respectively.

Expression of genes for MEL production by *P. antarctica* on wheat plant surface

The sensitivity of RT-PCR with SYBR Green I for expression analysis of each target gene was confirmed based on the correlation curves between cDNA amounts and C_t values (Table 1). Based on melt curve profiles obtained, no typical
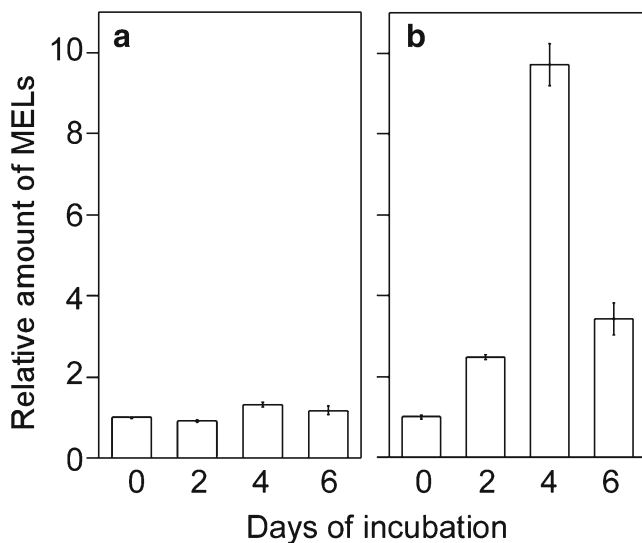
Fig. 6 Relative amount of MELs produced by *P. antarctica* cells incubated in water only (**a**) and water with wheat leaf segments (**b**). The amount of MELs in each ethyl acetate extract was quantified based on peak area in HPLC chromatogram. The amount of MELs in the supernatant of each suspension at 0 day was regarded as 1.0. *Each value* represents the average of three determinations and each *error bar* indicates the standard error of the mean

multiple Tm peaks formed on the cDNA amplicons were observed, and no or only little amplification of each target genes in the negative control samples were found; the averaged C_t values of the examined cDNA samples for actin gene, *PaEMT1*, and *PaMMF1* were 27.57, 26.14, and 30.76, respectively, while those of the observed negative controls were 32.89, 35.06, and 36.53, respectively. In the first trial (Table 1), although the differences were smaller compared to those in the second trial, the expression of *PaEMT1* was upregulated in the cells on wheat plant surface after 4, 6, and 8 days of incubation, showing a level of over 1.5-fold higher than that at 1 day after inoculation. Expression pattern of the MELs transporter gene, *PaMMF1*, was similar to that of *PaEMT1*, showing 1.74- to 3.13-fold increase in expression level. Similarly, in the second trial (Table 1), *PaEMT1* expression in the T-34 cells at 4,6, and 8 days after inoculation was higher compared to that at 1 day after inoculation, ranging from 2.21- to 3.46-fold. Expression of *PaMMF1* in the cells after 4, 6, and 8 days of incubation on wheat plant surface also increased with similar expression level. Based on the combined data of the two trials, the increased expression levels of *PaEMT1* and *PaMMF1* in the cDNA samples at 4, 6, and 8 days after inoculation were significantly different ($P<0.05$) from their respective expressions at 1 day after inoculation.

Discussion

In this study, comparison of cell morphologies between the wild type and *ΔPaEMT1* strains of *P. antarctica* on

hydrophobic plastic surface and onion peels indicated that MELs promoted morphological changes in fungal cells from yeast-like to filamentous form on solid surface. While morphological differences between these strains have been observed in liquid culture (Morita et al. 2010), our results confirmed that MELs have also a role on the morphological changes on solid surface. Like signal transduction pathways, functional roles of morphological change from yeast-like to filamentous form have been well studied in another basidiomycetous fungus, *U. maydis*; the filamentous growth induced with host plant-derived lipid (Klose et al. 2004; Klose and Kronstad 2006) has been reported to be necessary for pathogenesis (Klosterman et al. 2007).

In the present study, we observed the growth of *P. antarctica* strains inoculated on the leaf surfaces of rice and wheat, and found that the colonized areas of these strains have become enlarged and their morphologies have changed in the presence of MELs. Microscopic observation of the *ΔPaEMT1* propagules placed onto rice and wheat leaves revealed that they have significantly smaller colonized areas compared to those of MELs-producing wild-type strain after 3 days of incubation (Fig. 4). These results strongly suggest that MELs play a role in promoting fungal colonization on solid surfaces. However, it is still unclear whether MELs promote the colonization directly or not; further investigations are necessary to determine MELs mechanism of action. The observed morphological change from single yeast-like cells to hyphal form and the expansion of the area colonized by *P. antarctica* on plant surfaces and its MELs production may be closely related. Hyphal morphotype of yeasts has generally been known to be advantageous for colonization on plants (Gognies et al. 2006; Klose et al. 2004). Gognies et al. (2006) reported that pseudohyphal growth of yeast strains, such as *Saccharomyces cerevisiae*, on the surfaces of vineyard grapes (*Vitis vinifera* L.) is needed for the cells to colonize plant tissues. The results of this study have implied the possibility that a similar association between morphological change to hyphal growth and enhancement of colonization on solid surface also exists in the *Pseudozyma* species. Efficient dispersal from plant surface and rapid recolonization on the surface (termed as "growth") have been suggested to be important attributes for ecological adaptation for phylloplane inhabiting yeasts (Fonseca and Inácio 2006). Many fungal species belonging to Ustilaginales have been linked to production of glycolipids. For instance, *Pseudozyma* and *Ustilago* spp. produce MELs and/or cellobiose lipids (Feldbrügge et al. 2013; Kitamoto et al. 2002). The ubiquitous presence of *P. antarctica* in the phyllosphere (Allen et al. 2004; Boekhout 2011; Kitamoto et al. 2011; Morita et al. 2012) may be plausibly explained by their production of MELs or possibly other glycolipids that could have enhanced their growth on plant surfaces. Further experiments are necessary to prove the possibility.

Table 1 Relative expression of erythritol/mannose transferase gene (*PaEMT1*) and mannosylerythritol lipids transporter gene (*PaMMF1*) in *Pseudozyma antarctica* T-34 on wheat leaves by RT-PCR analyses

Days after inoculation	Trial 1			Trial 2		
	PaEMT1	*PaMMF1*	Actin gene	*PaEMT1*	*PaMMF1*	Actin gene
1	1	1		1	1	
2	1.17±0.12[a]	0.81±0.08		NT	NT	
4	2.14±0.33	2.61±0.04		2.21±0.32	5.74±4.11	
6	1.55±0.18	1.74±0.21		3.46±0.86	5.60±2.31	
8	2.92±0.59	3.13±0.49		2.61±0.49	4.26±1.57	
(Calibration curve)						
Slope	−3.38±0.06	−3.68±0.07	−3.57±0.06	−3.46±0.03	−3.40±0.07	−3.38±0.13
Y-intercept	18.14±0.33	21.31±0.39	21.56±0.29	21.34±1.53	17.12±0.10	16.91±0.52
R^{2b}	0.997±0.001	0.997±0.001	0.999±0.000	0.999±0.000	0.995±0.003	0.993±0.001
Eff (%)[c]	97.96±2.53	87.17±2.14	90.57±2.03	94.60±1.19	97.14±2.85	98.69±4.95

The gene expression was normalized based on actin gene expression and the expression in the cells at 1 day after inoculation was regarded as 1

NT not tested

[a] Mean value ± standard error of triplicate analyses

[b] Coefficient of determination of calibration curve

[c] PCR efficiency

Production of MELs by *P. antarctica* in the presence of *Gramineae* leaf was confirmed by HPLC analysis; the amounts of the compounds increased in the suspension containing wheat leaf segments, indicating that the production of MELs by *P. antarctica* is promoted de novo in the presence of the leaf segments. However, the relative amount of MELs in the extract of cell suspension with leaf segments after 6 days of incubation was found to decrease compared to that after 4 days of incubation. This result could possibly be due to the assimilation of these compounds by *P. antarctica* as a nutrient source for growth during the incubation period. The utilization of MELs by the producers has not been elucidated in vitro and in planta. Further verifications are necessary to ascertain this hypothetical role of the compounds. In addition, we also tried to detect MELs secreted by *P. antarctica* on wheat and rice leaves by TLC and HPLC analyses as described in a previous report (Morita et al. 2006), but MEL was not clearly observed on the chromatograms due to the presence of impurities in the extracts that masked the specific detection of MELs (data not shown). For effective direct detection of the compounds produced by *P. antarctica* on leaves, it is necessary to improve the purification procedures. On the other hand, RT-PCR analyses revealed that the expression level of both MELs genes of *P. antarctica*, erythritol/mannose transferase gene (*PaEMT1*) and a putative MEL transporter gene (*PaMMF1*), were both higher in the cells on wheat leaves at 4, 6, and 8 days after inoculation compared to those in 1 day-incubated cells (Table 1). These results support the idea that *P. antarctica* has a potential to produce these compounds on leaves. The expression of *PaEMT1* and *PaMMF1* genes showed synchronized

patterns. The former gene has been recognized to function during the early stage of the MEL synthetic pathway, while *PaMMF1* is considered as the gene responsible for MELs secretion (Morita et al. 2013). This implies that MELs generated in the cells may be promptly secreted outside the cells on the leaf surface.

Although *Pseudozyma* spp. have been isolated from various phyllospheres, a previous report (Clément-Mathieu et al. 2008) stated that a *P. antarctica* strain inoculated on the surface of cucumber leaves did not grow. This result may be attributed to the unsuitable nutritional conditions in the cucumber plant adversely affecting the growth of the test strain. On the other hand, *Gramineae* plants seem to harbor this fungal species well (Allen et al. 2004; Kitamoto et al. 2011; Morita et al. 2012), implying that their plant surfaces contain nutrients favorable for the species to grow and produce MELs. Nutritional conditions for MELs production by *Pseudozyma* spp. have been well elucidated in liquid culture (Kitamoto et al. 2002; Morita et al. 2006; Rau et al. 2005). *P. antarctica* produces MELs from vegetable oils and from glucose as sole carbon source in liquid cultures (Kitamoto et al. 2002; Morita et al. 2007); the yields of the compounds were significantly higher with the former carbon sources than with the latter one (Morita et al. 2007). Plant leaf surfaces have generally been recognized to be nutrient-limited environment (Andrews 1992), with their actual compositions and concentrations varying depending on plant species, leaf age, and growth conditions (Fonseca and Inácio 2006); they include diverse carbons derived from endogenous and exogenous origins of the plants (Andrews 1992). We hypothesized that *P. antarctica* may use

them to produce MELs. Particularly, derivatives of cuticle of the leaf surface may be utilized as a carbon source for the fungal growth. It is well known that cuticles are produced by most terrestrial plants, and that they consist of cutin, a mixture of fatty and hydroxyl acids and cuticular waxes comprising of long chain hydroxycarbons and alkyl ester, etc. (Andrews 1992; Kitamoto et al. 2011). *Pseudozyma* spp. have been reported to secrete various hydrolytic enzymes (Kitamoto et al. 2002; Seo et al. 2007). Indeed, we have recently found that the *P. antarctica* strains isolated from various leaf surfaces degraded biodegradable plastics whose chemical structures closely resemble cutin (Kitamoto et al. 2011). These indicate the possibility that hydrocarbons derived from cuticle, which are presumably similar to those derived from vegetable oils, can also trigger the production of MELs. Experiments to prove the hypothesis, as well as to verify temporal dynamics of production of both hydrolytic enzymes and MELs, are ongoing. Furthermore, the possibility that the fungus produced the biosurfactants using other nutrients, such as leakages from inside the leaves through wounds, etc. also remains. Further elucidations are necessary to ascertain the impact, if any, of internal nutrients of wheat leaves on the production of MELs.

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