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Methanol bioeconomy: promotion of rice crop yield in paddy fields with microbial cells prepared from natural gas-derived C_1 compound

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Summary

Methylotrophs, which can utilize methanol as a sole carbon source, are promising microorganisms to be exploited in a methanol-based bioeconomy, in which a variety of useful compounds are biotechnologically produced from natural gas-derived methanol. Pink-pigmented facultative methylotrophs (PPFMs) are common plant phyllospheric bacteria and are known to enhance seedling growth and total biomass of various plants. However, improvement of crop yield by inoculation of PPFMs at the field level has not been well investigated. We herein describe improvement of crop yield of several rice cultivars by foliar spraying of PPFMs. After selection of PPFM strains and rice cultivars by the *in vitro* seedling growth test, we further conducted paddy field experiments. The crop yield of the sake-brewing rice *Oryza sativa* cultivar Hakutsurunishiki was reproducibly improved in a commercial paddy field for over a 5-year period. A one-time foliar spray of PPFM cells (living or killed) or a cell wall polysaccharide fraction, after the heading date, acted in the phyllosphere and effectively improved crop yield. Our results show that the established process with PPFMs is feasible for improvement of food production in the methanol bioeconomy.

Introduction

Methanol, which is industrially produced from natural gas, has been a promising carbon resource for chemical and biotechnological processes. A methanol-based economy has also been proposed, in which methanol is used as a fuel or a feedstock for various chemicals to replace fossil fuels (Olah *et al*., 2009; Olah, 2013).

Methylotrophs are microorganisms that can utilize C_1 compounds such as methanol and methane as the sole source of carbon and energy. Methanol-utilizing methylotrophs have been highly exploited for production of biochemicals such as coenzymes [coenzyme Q (CoQ) and pyrroloquinoline quinone (PQQ)], ATP, heterologous proteins and polyhydroxyalkanoates (Natori *et al*., 1978; Urakami *et al*., 1992; Cregg *et al*., 1993; Sakai *et al*., 1994; Schrader *et al*., 2009; Ochsner *et al*., 2015). The carbon resource for the production of methylotrophic cells and the bioproduction process from methanol as a raw material does not compete with other edible carbon resources such as sugar or organic acids. During the 1970s, industrial-scale production of single-cell proteins (SCPs) as an economical livestock feed was established with methylotrophs (Windass *et al*., 1980). This was based on high-cell density cultivation of methylotrophs grown on methanol as the sole carbon source.

In the last decade, methylotrophs have attracted much attention as microbes that participate in a novel type of microbe–plant interaction, i.e. symbiosis at the phyllosphere (surfaces at the above part of plant) (Vorholt, 2012). Pink-pigmented facultative methylotrophs

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(PPFMs), e.g. *Methylobacterium* spp. and *Methylorubrum* spp., are known to ubiquitously inhabit and dominate the microbial community on plant leaves, utilizing methanol as a carbon source (Knief *et al.*, 2010; Knief *et al.*, 2012). The surface area of plant leaves (both sides total) is estimated to be 1.0 x 10^9 km^2, which corresponds to more than six times the land area on earth. The number of PPFMs on leaves has been estimated to be 10^{25}–10^{27} cells or by mass weight 10^7–10^9 t. The amount of global methanol emitted from plants that escapes methylotrophs and is released into the atmosphere has been estimated to be *ca.* 10^8 t $year^{-1}$ (Stavrakou *et al.*, 2011; Messina *et al.*, 2016; Henrot *et al.*, 2017). Therefore, methanol conversion to biomass by PPFMs affects the carbon cycling between two major greenhouse gases, methane and CO_2. The methylester group of pectin, a ubiquitous plant cell wall component, is considered to be the major source of methanol in the phyllosphere (Fall and Benson, 1996). Methylotrophs have been found to adapt to diurnal environmental changes on plant leaves, i.e. methanol concentration, temperature and UV/light (Kawaguchi *et al.*, 2011; Iguchi *et al.*, 2018). However, distinct from the well-studied microbe–plant interactions in the rhizosphere (soil environment), many basic scientific questions regarding microbe–plant interactions in the phyllosphere remain to be solved, and the practical use of synergistic interactions between methylotrophs and plants in agriculture is expected to be explored in the future.

Past studies have demonstrated that plant seeds inoculated with PPFMs show enhanced plant growth, i.e. enhancement of germination rate, seedling growth and total biomass (Abanda-Nkpwatt *et al.*, 2006; Ryu *et al.*, 2006; Meena *et al.*, 2012). If such positive effects of PPFM–plant interactions can be induced in the phyllosphere, 'foliar spraying of PPFMs' would be an effective mean to increase biomass and food production in agriculture. Indeed, the effectiveness of foliar spray of PPFMs was reported for the growth promotion of cotton and sugarcane (Madhaiyan *et al.*, 2006). Convenient distribution of PPFMs together with pesticides is possible from the air by a drone and this sort of application of microbes in precision farming has been recently reported (Bharathi,*et al.*, 2017; Compant *et al.*, 2019; Preininger *et al.*, 2018). Since PPFMs could reach and act on the aerial parts of the plant surface, the amount of PPFMs would be minimized by avoiding dilution of PPFMs into soil or paddy water. The number of times and the timing of foliar spraying, and the amount of PPFMs can be easily controlled.

In the previous studies, inoculation of PPFMs promoted seedling growth of vegetables under laboratory conditions or in pot-scale cultivation (Ryu *et al.*, 2006; Madhaiyan *et al.*, 2010; Tani *et al.*, 2012; Kumar *et al.*, 2016). Combinations of seed inoculation and foliar spray treatment increased rice grain yields in greenhouse pot culture experiments (Madhaiyan *et al.*, 2004). However, our previous trial in a rice paddy with seed inoculation of PPFMs resulted in no or negative effects on rice grain yields (Tani *et al.*, 2015). Furthermore, inoculated PPFM strains could not be detected from the mature plants. Assessment of rice grain yields in the rice paddy suffers from physical and temporal constraints; each experimental condition requires sufficient rice paddy area, and only one trial can be completed per year. Yearly changes in the environment (mostly due to climate conditions) have hindered re-examination or reproduction of the experiments.

Here, we tested and optimized the timing of PPFM inoculation to a Japanese sake-brewing rice cultivar in a commercial paddy field and found that foliar spraying of not only living PPFM cells, but also killed cells or a cell wall polysaccharide fraction improved crop yields. These results show that foliar spraying of PPFMs is feasible for use in agriculture as part of the methanol bioeconomy, in which a variety of useful compounds are biotechnologically produced from natural gas- or biomass-derived methanol.

Results

Screening for PPFM strains that have growth-promoting effects on rice seedling

First, 10 PPFM strains that improved the germination rate of rice seeds and the length of roots and shoots of rice seedlings were selected from 45 candidate PPFM strains isolated from various plant-associated samples, e.g. plant leaves, rice and paddy water through enrichment culture on methanol medium. The growth-promoting activity on the rice seedlings was evaluated *in vitro*. We used the rice (*Oryza sativa*) cultivar Koshihikari, which is the most popular rice cultivar in Japan. Surface-sterilized rice seeds were treated with a cell suspension of each PPFM strain and cultivated on cotton under aseptic conditions for 6 days. Among the tested strains, the shoot lengths of the seedlings treated with strains C1, Daff, RS1, Rst and Sh were significantly longer than those of the untreated control (Fig. 1A). The root lengths of the seedlings treated with strains Daff, Rfw, Rst and Sh were significantly longer than those of the control (Fig. 1B). Strains Daff, Rst and Sh therefore positively affected both shoot and root lengths.

The 16S rRNA gene sequencing of the 10 PPFM strains revealed that 7 and 3 strains belong to the genera *Methylobacterium* (*Mb*) and *Methylorubrum* (*Mr*) respectively (Table S1). The phylogenetic tree showed that strains RS1, Sh, By, B2B and In1 were closely related to *Mb. fujisawaense,* strains C1 and Daff were

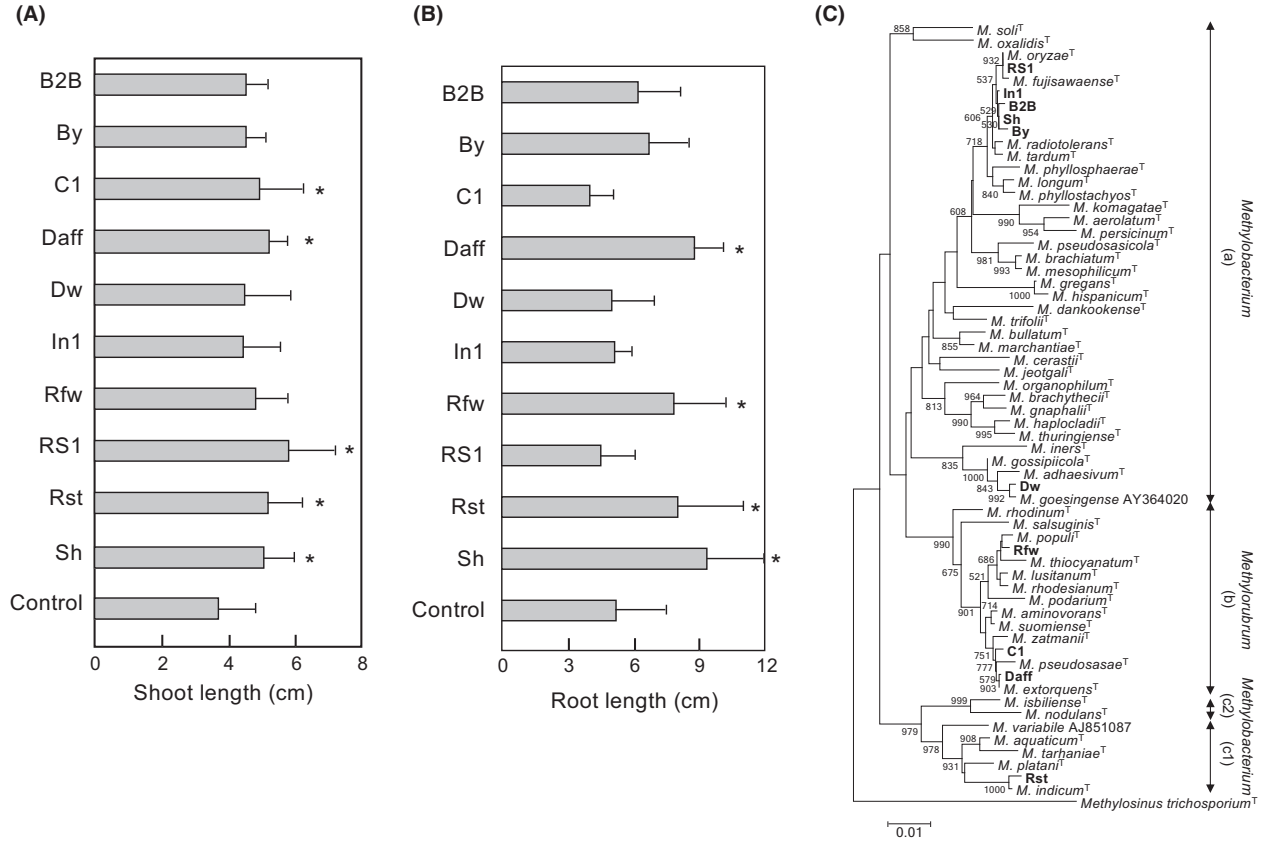

Fig. 1. Effect of treatment with PPFM isolates on growth of rice seedings (cultivar Koshihikari) and phylogenetic analysis of the isolates. Sterilized rice seeds were sown in PPFM cell suspension or water (control), and cultivated for 6 days on dampened cotton under aseptic conditions. The test was started with 30 seeds, and the shoot length (A) and root length (B) of the grown seedlings were analysed (n = 11–23). Data are presented as means and error bars represent standard deviations. Statistically significant difference between control and treatment was assessed using one-way ANOVA (*P < 0.05). C. Phylogenetic tree of the PPFM strains isolated from plants and *Methylobacterium* and *Methylorubrum* type strains. The bootstrap values > 500 (from 1000 replicates) are presented at the corresponding nodes. The bar indicates 1% sequence dissimilarity. Grouping of PPFM species was based on Green and Ardley (2018).

closely related to *Mr. extorquens*, and the other three strains Rst, Rfw and Dw were located in different clusters in the phylogenetic tree (Fig. 1C).

Some plant-associated bacteria are known to produce phytohormones such as auxin, and the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, to reduce ethylene levels in plants (Ortiz-Castro *et al.*, 2009). Next, 10 PPFM isolates were characterized based on properties related to plant growth regulation, i.e. production of indole acetic acid (IAA), ACC deaminase, urease and activity of phosphate solubilization and nitrogen fixation (Table S1); these properties were considered in selecting PPFM strains as described below.

Effect of PPFM seed inoculation on growth of seedlings of various rice cultivars

The results described above were examined with the rice cultivar Koshihikari. To determine the effect of PPFM on other rice cultivars, we examined whether inoculation of PPFM could promote seedling growth of 48 rice cultivars from the rice core collection of Japanese landraces, together with Koshihikari as a control cultivar. In nature, various PPFMs live in the phyllosphere, and we have observed that some plant hosts show specificity to PPFMs for colonization (Mizuno *et al.*, 2012; Mizuno *et al.*, 2013). Therefore, in this trial, we used a mixture of 3 PPFM strains, RS1, Rst and Sh, to screen for appropriate combination of PPFM and host rice cultivar. In the phylogenetic tree (Fig. 1C), strains RS1 and Sh are closely related, whereas strain Rst is located far from the other two strains. All three strains positively affected shoot length, and strains Rst and Sh gave positively affected root length (Fig. 2A and B). Among these three strains, strain Rst produced a detectable level of IAA (Table S1). If interactions, positive or negative, exist between PPFM and some rice cultivars even in the presence of other ineffective or negative PPFM strains, we

expected that we could obtain PPFM strains showing positive effects on the growth of rice seedlings.

Rice seeds were treated with a cell suspension of the PPFM mixture and cultivated on vermiculite under open-air (non-aseptic) conditions mimicking natural cultivation conditions. Treatment with the PPFM mixture significantly enhanced the shoot lengths of 30 and root lengths of eight rice cultivars, respectively, by 10–70% (Table S2). Dry weights of 21 cultivars significantly increased in response to PPFM treatment. Negative effects of the PPFM treatment were also observed on the shoot length of one cultivar and the root lengths of two cultivars (Table S2).

The eight rice cultivars JRC-17, JRC-19, JRC-21, JRC-23, JRC-32, JRC-33, JRC-34 and JRC-54, which exhibited higher growth promotion effects, were subjected to a second trial to reproduce the positive effects (Fig. 2). Consistent with the first trial (Table S2), rice cultivars JRC-19, JRC-32, JRC-33, JRC-34 and JRC-54 had positive effects on shoot length and dry weight of the plant. Among these cultivars, JRC-32 (Omachi) and JRC-33 (Shinriki) exhibited strong positive effects not only on the shoot length but also on the dry weight (Fig. 2). These cultivars are known as Japanese sake-brewing rice cultivars.

These *in vitro* experiments showed that PPFM treatment could improve the lengths of shoots and roots, and the dry weight of the host plant after 7 days of germination. However, the extent of the PPFM effects differed between host rice cultivars.

Paddy field trial with the PPFM mixture (2013)

Paddy field trials were conducted in two geographically distant areas of Japan, Kyoto prefecture and Okayama prefecture. Rice seedlings of three cultivars, JRC-32 (Omachi), JRC-33 (Shinriki) and Koshihikari, were prepared from seeds treated with the PPFM mixture and transplanted to the paddy fields (*ca*. 60 m^2). Rice plants grown in the paddy fields were further treated with the PPFM mixture by foliar spraying.

The PPFM-treated rice seedlings were transplanted to a rice paddy in Kyoto on 19 June 2013, and the shoot lengths of rice plants were measured during the growing stage. After a month (on 17 July 2013), before foliar spraying, the PPFM-treated Shinriki cultivar showed significantly longer shoot lengths than the untreated one (Fig. 3). On 27 August, 7 days after the third foliar spraying, the shoot lengths of all three cultivars treated by the PPFM mixture showed higher average values by 2–4 cm than those of untreated cultivars (Fig. 3).

The results of the harvested rice yield are shown and summarized in Fig. S1 and Table 1. Cultivar Shinriki treated by the PPFM mixture exhibited increased values in all measured indexes both in Kyoto and Okayama paddy fields compared to the untreated plants (Fig. S1). The PPFM treatment strongly affected the grain yields of Shinriki, i.e. the number and weight of grains were increased by 24% and 28% in Kyoto and 8.2% and 12% in Okayama, respectively, with an increase in the number of panicles (Table 1). On the other hand, PPFM treatment of Koshihikari significantly reduced the dry weight of plants as well as the number of panicles and grains, and the weight of grains cultivated in Okayama (Table 1). In the case of Omachi, the PPFM treatment resulted in statistically significant decreases in all values in Kyoto and Okayama (Fig. S1).

The dry weight of plants was affected for all three cultivars. The differences between untreated and PPFM-treated conditions were likely reflected in the differences of grain weight (Fig. S1A and D). These results indicated that the PPFM mixture negatively affected the grain ripening of Koshihikari and Omachi cultivars and had a positive effect on the grain yield of the Shinriki cultivar.

Crop yield increase of Sake-brewing rice cultivars by foliar spraying of strain Rst in a commercial paddy field (2014-2018)

Trials with living PPFM cells (2014–2016): the timing of foliar spraying. In order to verify the effects of PPFM inoculation on the crop yield of sake-brewing rice in a commercial paddy field and to establish an economical method for foliar spraying, we used the commercial sake-brewing rice cultivar Hakutsurunishiki, and PPFM foliar spray trials were conducted in commercial paddy fields (*ca*. 200 m^2) located in Hyogo Prefecture, Japan, from 2014 to 2018. Crop yield data were obtained from *ca*. 100 hills of rice plants each year.

In 2014, the weight of brown rice decreased after foliar spraying with the PPFM mixture regardless of the number of sprayings, compared to that of the untreated control (Table 2). In the 2015 trial, we tested seed inoculation and foliar sprays of the PPFM mixture and with strain Rst alone, which was the highest producer of IAA among the tested PPFMs (Table S1). Apparently, PPFM inoculation enhanced greening of rice seedling leaves before transplantation [Fig. S2; 3 June 2015 (14 days after sowing)]. This leaf greenization can be ascribed to the increase of the plant chlorophyll content by PPFM inoculation as recently reported (Madhaiyan *et al*., 2015; Krishnamoorthy *et al*., 2020). While the weight of brown rice decreased in the tested area with seed inoculation, foliar spraying with the PPFM mixture or strain Rst without seed inoculation increased the weight of brown rice (Table 2). Judging from the results of 2014 and 2015, seed inoculation and foliar spraying during the early stages of growth had repressive effects

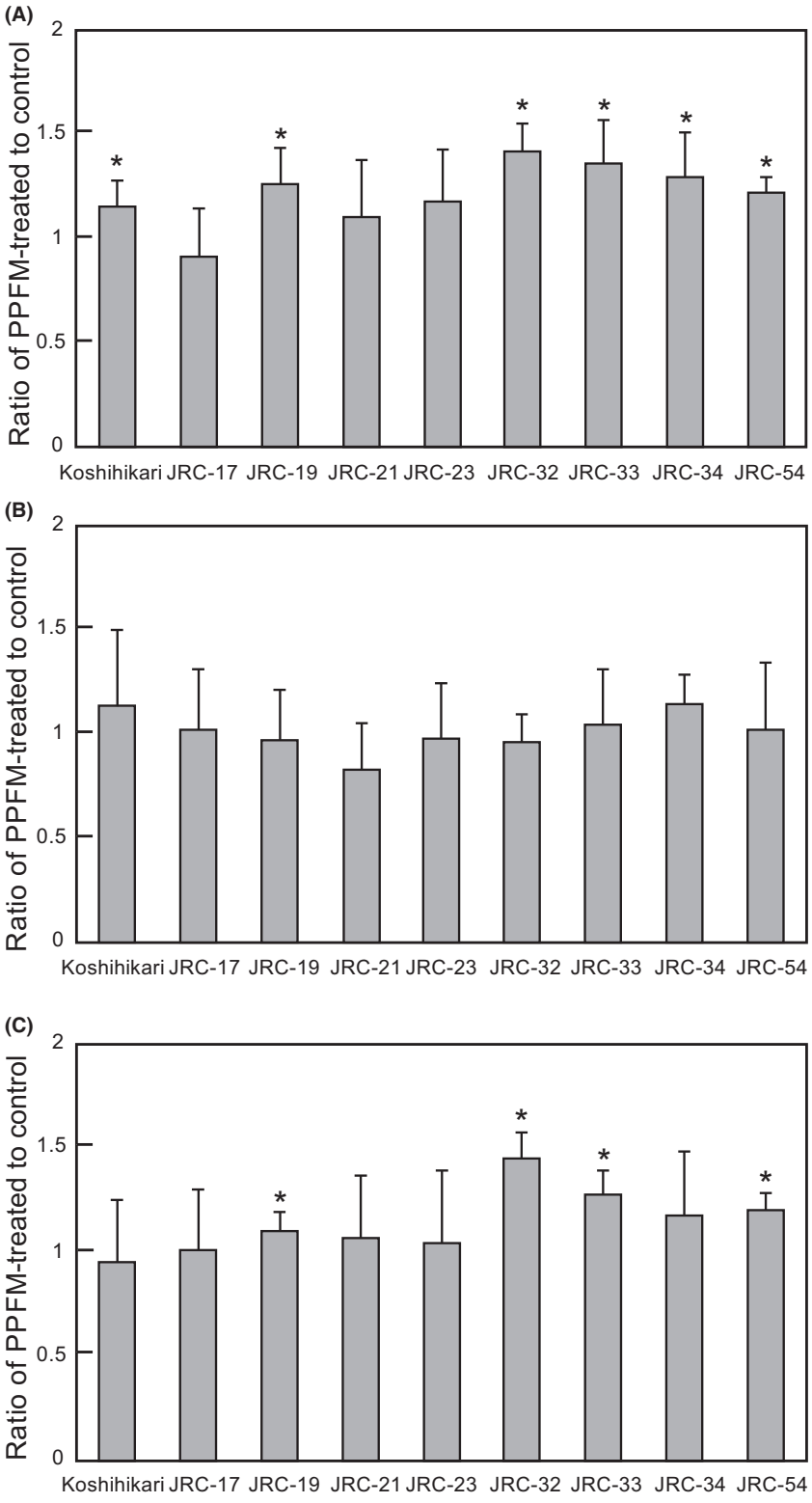

Fig. 2. Effect of treatment with the PPFM mixture on various rice cultivars. Nine cultivars selected from the results of the first test shown in Table S1 were subjected to this test. Rice seeds were sown in a cell suspension of three PPFM strains, RS1, Rst and Sh, or water (control), and cultivated for 7 days on vermiculite under open air. The values indicate the ratio of PPFM-treated to uninoculated (water-treated control). The test was started with 10 seeds, and shoot lengths (A), root lengths (B) and dry weight of seedlings (C) of grown seedlings were analysed (n = 8–10). Data are presented as means, and error bars represent standard deviations. Statistically significant difference between control and treatment was assessed using the t-test (*$P < 0.05$).

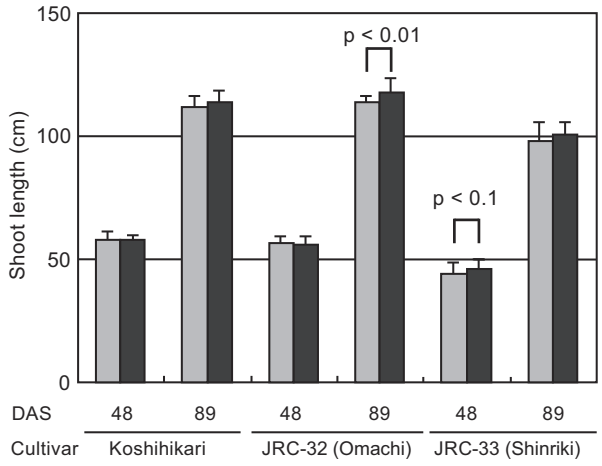

Fig. 3. Shoot length of rice plants cultivated in the paddy field in Kyoto. The distance from the water surface to the top position of shoots for each plant was measured on indicated days after sowing (DAS). Data are presented as means and error bars represent standard deviations (n = 18). Grey bars, uninoculated control; black bars, PPFM-treated. Statistically significant difference between control and treatment was assessed using the t-test.

on crop yield. In contrast, foliar spraying of strain Rst before and/or after the heading date had positive effects on crop yield. In 2016, the increase of the weight of brown rice was reproduced by foliar spraying of strain Rst before and after heading date (Table 2).

Trials with autoclave-killed PPFMs and the cell wall fraction of PPFM cells (2017-2018). Since living PPFM cells could proliferate in nature and change the environmental ecology, foliar spraying of killed PPFM cells or active compounds would be more favourable for storage and biosafety.

We speculated that foliar spraying before and after the heading date would affect the plant during the ripening period and translocation. In the 2017 and 2018 trials, the effectiveness of foliar spraying of strain Rst was further investigated using living cells, together with autoclave-killed cells, and the cell wall polysaccharide fraction from strain Rst. In the 2017 trial, the weight of brown rice from the rice plants treated by foliar spraying with living cells, killed cells and the cell wall polysaccharide fraction of strain Rst increased 2–4% relative to that by foliar spraying of the spreading agent as a control (Table 3). Thus, not only living cells but also killed cells, and the cell wall polysaccharide fraction, had positive effects on crop yield by foliar spraying before and after the heading date. In the 2018 trial, we tested foliar spraying of killed cells of strain Rst before and/or after the heading date. The weight of brown rice from the rice plants treated by killed cells of strain Rst increased 1–7% relative to that by the foliar spraying of the spreading agent as a control, and foliar spraying after the heading date was more effective than that before the heading date (Table 3). Furthermore, the rate of ripening was significantly increased by foliar spraying with killed cells, and the unit yield from the plants treated by the foliar spraying with killed cells after the heading date increased 16% relative to the control (Table 3). These results indicate that foliar spraying of killed cells of strain Rst after the heading date was most effective in increasing of the rate of ripening, resulting in an increased of crop yield of the rice cultivar Hakutsurunishiki.

Discussion

Our trials in the commercial paddy field revealed that foliar spraying of PPFM after the heading date had significant positive effects on ripening and crop yield of the sake-brewing rice cultivar Hakutsurunishiki (Table 3). Although the foliar spraying of PPFM at the plant growing stage enhanced shoot length and leaf greenization (Fig. 3 and S2), the final crop yield was decreased. On the other hand, even foliar spraying of killed PPFM or the cell wall polysaccharide fraction after the heading date was found to increase the crop yield of rice (Table 3). These results suggest that the positive effects were not the result of nutrients supplied to plants, but a direct stimulation by the cell wall components of PPFM during the translocation stage of rice plants. We still do not know the underlying mechanism of the positive effects of the microbes or their components after the heading date of rice. However, the species-specific

Table 1. PPFM treatment effects on crop yields in paddy field experiments.

Cultivar	Plantation	Dry weight of plant	Number of panicles	Number of grains	Weight of grains	1000 grains weight
Koshihikari	Kyoto	+0.14%	–4.0%	–1.8%	+1.3%	+3.1%
	Okayama	–15%	–12%	–12%	–16%	–3.7%
JRC–32	Kyoto	–5.2%	–0.81%	–9.8%	–15%	–5.2%
(Omachi)	Okayama	–17%	–21%	–18%	–22%	–4.9%
JRC-33	Kyoto	+15%	+8.4%	+24%	+28%	+3.8%
(Shinriki)	Okayama	+9.9%	+8.4%	+8.2%	+12%	+3.0%

Values are expressed as the percentage change, which was calculated from the ratio of the average value of PPFM-treated rice to that of uninoculated rice (Fig. S1).

Table 2. Effects of PPFM treatment and foliar spraying on rice yields (cultivar Hakutsurunisiki) in a commercial paddy field from 2014 to 2016.

Year	Test area No.	Treatment	Date of foliar spraying (days after sowing)				Weight of brown rice (kg a^{-1})	Relative weight of brown rice (%)[a]
2014			29 May (13)	14 Jul. (59)	5 Aug. (81)	8 Sep. (115)		
	1	Control (no treatment)	–	–	–	–	68.0	100
	2	Foliar spraying with the PPFM mixture[b]	+	–	–	–	65.1	95.8
	3		+	+	–	–	64.0	94.1
	4		+	+	+	–	53.1	78.2
	5		+	+	+	+	55.7	81.9
2015			7 Aug. (79)			14 Sep. (117)		
	1	Control (no treatment)	–			–	62.7	100
	2	Seed inoculation[c] and foliar spraying with the PPFM mixture	+			+	44.2	71.0
	3	Seed inoculation and foliar spraying with strain Rst	+			+	40.2	64.0
	4	Foliar spraying with the PPFM mixture	+			+	68.7	110
	5	Foliar spraying with strain Rst	+			+	68.7	110
2016			8 Aug. (81)			16 Sep. (120)		
	1	Control (no treatment)	–			–	42.2	100
	2	Foliar spraying with strain Rst	+			+	49.3	117
	3	Foliar spraying with killed cells of strain Rst	+			+	53.2	126

a. Relative values are shown as a percentage of the control treatment in each year.
b. The PPFM mixture contains strains RS1, Rst and Sh.
c. Seeds were inoculated with strains By, RS1 and Sh.

Table 3. Effects of PPFM treatment on rice yields (cultivar Hakutsurunisiki) in a commercial paddy field in 2017 and 2018.

Year	Test area No.	Treatment	Date of folia spraying (Days after sowing)		Weight of brown rice (Kg a^{-1})	Relative weight of brown rice (%)[a]	Rate of ripening (%)	Unit yield (g m^{-2})[b]	Relative unit yield (%)[a]
2017			8 Aug. (80)	6 Sep. (109)					
	1	Control (foliar spraying of the spreading agent)	+	+	68.1	100	n.t.	n.t.	n.t.
	2	Foliar spraying with living cells of strain Rst	+	+	69.6	102	n.t.	n.t.	n.t.
	3	Foliar spraying with killed cells of strain Rst	+	+	69.4	102	n.t.	n.t.	n.t.
	4	Foliar spraying with the cell wall polysaccharide fraction of strain Rst	+	+	70.7	104	n.t.	n.t.	n.t.
2018			9 Aug. (81)	14 Sep. (117)					
	1	Control (foliar spraying of the spreading agent)	+	+	63.1	100	75.3	561	100
	2	Foliar spraying with killed cells of strain Rst	+	+	66.3	105	82.0	652	116
	3	Foliar spraying with killed cells of strain Rst	+	-	63.9	101	81.7	591	105
	4	Foliar spraying with killed cells of strain Rst	-	+	67.6	107	80.3	648	116

n.t., not tested.
Relative values are shown as a percentage of the control treatment in each year.
b. Unit yield (g m^{-2}) = number of panicles (/m^2) x number of grains per panicle x rate of ripening x weight of one grain (g).

structure of the bacterial cell wall may explain the differing effects on crop yields among rice cultivars. Thus, foliar spraying could be a novel approach for using microbes in agriculture, as PPFMs can be easily used as spraying agents together with pesticides, distinct from the use of microbial fertilizers or rhizosphere microorganisms functioning in soil.

The agricultural use of microbes has been greatly hampered by the culture cost for production of microbial cells, in which molasses sugars or organic acids are used as conventional carbon sources. Distinctively, PPFMs can be cultivated at high-cell density with methanol as a carbon source, collected and killed at a large industrial scale. Among the three strains, Rst, RS1 and

Sh, strain Rst gave the best growth yield on methanol medium. According to our estimation, three batch cultures of 2-t scale high-cell density cultivation would be sufficient to provide PPFM cells to cover all of the paddy fields in Japan, and the process is economically feasible. Also, the bioactive component polysaccharide cannot be produced by chemical synthesis even after elucidation of its chemical structure.

In the established PPFM fermentation process, a natural gas-derived carbon source is converted to PPFM biomass as a strategy to increase of rice production. The carbon flow represents the utilization of two representative greenhouse gases, CO_2 and methane, a main component of natural gas. Therefore, large-scale application of PPFM foliar spray will lead to great impact with respect to the environmental aspect of the methanol bioeconomy.

Due to an acute increase of Japanese sake exports from Japan (doubled in the last two decades) and the shortage of paddy fields and farmers to grow sake-brewing rice, the demand for production of sake-brewing rice has been increasing. However, cultivation of sake-brewing rice has already been optimized to achieve the best grain yield by introducing improvements in fertilization and control of paddy water, and the required empirical skills of farmers. Therefore, it has been difficult to develop a stable technology to further improve rice grain yield. In this trial, we applied PPFM to the sake-brewing rice cultivar Hakutsurunishiki, since we had access to commercial paddy fields and skilled farmers. The harvested PPFM-treated rice was subjected to fermentation tests for sake, and compared to the control rice, no differences in the protein or lipid contents or in the flavours and taste of the sake were detected. In the 2019 trial, improvement of Hakutsurunisiki yield by PPFM treatment was reproduced in larger paddy fields located in different rice paddy of Hyogo prefecture (*ca*. 600 m^2). It is noteworthy that the rice grain yield was further increased in a large-scale commercial rice paddy with a foliar spraying approach during transportation of the rice plants. Similar approaches to increase the crop yield should be possible for other rice cultivars, and possibly other major crops.

Experimental procedures

Isolation of PPFMs

Plant leaves, rice samples and paddy water were collected and incubated at 28°C with shaking in nitrate mineral salt (NMS) medium (Whittenbury *et al*., 1970) supplemented with 0.5% methanol as a carbon source and 0.01% yeast extract. The 4-day cultivation broth was diluted and spread on NMS agar plates containing 0.5% methanol. Pink colonies were isolated.

Phylogenetic analysis

The 16S rRNA gene from each bacterial isolate was amplified by PCR using the 27f-1492r primer set (Weisburg *et al*., 1991) and cloned into the plasmid pMD20 (TakaraBio, Shiga, Japan) by the TA-cloning strategy. The 16S rRNA gene on each plasmid was sequenced. The sequences (*ca*. 1500 bp) were aligned using the CLUSTAL W program (Thompson *et al*., 1994), and a phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with Kimura model (Kimura, 1980). The 16S rRNA gene sequences of the PPFM isolates have been deposited under accession numbers LC552134–LC552143.

Biochemical characterization

Isolates were tested for the production of IAA (Rodrigues *et al*., 2008) and siderophore (Schwyn and Neilands, 1987), and the ability to solubilize phosphate (Nautiyal, 1999). For the IAA assay, PPFM strains were cultured in the NMS medium containing 1 mM L-tryptophan. The enzyme activities of urease (Madhaiyan *et al*., 2010) and ACC deaminase (Penrose and Glick, 2003) were also examined. The ability to fix nitrogen was tested by growth on nitrogen-free NMS medium and subsequently confirmed by PCR amplification of the *nifH* gene using universal primers (Poly *et al*., 2001).

In vitro seedling growth test with PPFM strains

Rice seeds (*Oryza sativa* Japonica cultivar Koshihikari) were sterilized by soaking in 70% ethanol for 1 min, followed by immersing in 30% commercial bleach solution containing 0.02% Tween 20 for 30 min and rinsing five times with sterilized water. PPFM cells were harvested from NMS liquid cultures by centrifugation, washed with water and suspended in water to a final OD_{600} = 1. Sterilized rice seeds were soaked in the bacterial suspension for 5 h at 28°C and then sown on sterilized cotton dampened with water in a glass bottle with a silicon cap. Uninoculated (water-treated) seeds were also prepared as the control. After incubation for 6 days in a plant growth chamber (Nippon Medical & Chemical Instruments, Osaka, Japan) at 25°C (14 h light/10 h darkness), the lengths of shoots and roots were measured.

In vitro seedling growth test of the Japanese rice collection with the mixture of PPFMs

The rice core collection of Japanese landraces was obtained from the National Agriculture and Food Research Organization (Ebana *et al*., 2008). To remove fungi, rice seeds were soaked in 0.5% (w/v) Benlate-T

solution (Sumitomo Chemical, Tokyo, Japan) overnight at 28°C and then rinsed with sterilized water.

Methylobacterium sp. strains RS1, Rst and Sh were grown in R2A liquid medium (Nihon Pharmaceutical, Tokyo, Japan) supplemented with 0.5% methanol. Cells were harvested by centrifugation, washed with water and suspended in water to a final OD_{600} = 1. An equal volume of each strain suspension was mixed to prepare the PPFM mixture. The seeds were soaked in the mixture for 2 h.

Vermiculite (1.5 kg) was mixed with 5 l of two thousand-fold diluted liquid fertilizer, Hyponex (Hyponex, Osaka, Japan), and sterilized by autoclaving for 60 min. This soil (*ca*. 12 g dry weight) was put in plastic pots, and two rice seeds were sown in each. The pots were placed in a plastic container containing tap water. After incubation for 7 days in a plant growth chamber at 28°C (14 h light/10 h darkness), the lengths of shoots and roots were measured. After drying at 80°C, the dry weight of seedlings was measured.

Paddy field experiments in Kyoto and Okayama

The rice seeds of cultivars Koshihikari, Omachi and Shinriki, treated with Benlate-T were soaked for 2 h in the PPFM mixture (strains RS1, Rst and Sh) prepared as described above and then sown on cotton dampened with water. After incubation in an incubator at 28°C, germinated seedlings were transplanted to pots containing culture soil and vermiculite. The seedlings were grown in the greenhouse for two or 3 weeks.

The rice cultivation was conducted in two experimental paddy fields in Kyoto (Graduate School of Agriculture, Kyoto University, 35°01'58"N 135°46'58"E) and Okayama (Institute of Plant Science and Resources at Okayama University, 34°35'29"N 133°46'09"E) prefectures in 2013. In the paddy field in Kyoto, plant spacing was 10 x 30 cm, and N, K_2O and P_2O_5 fertilizers were applied in quantities of 6, 9 and 9 kg 100 m^{-2} respectively. In the paddy field in Okayama, plant spacing was 20 x 30 cm, and fertilizer was not applied. In both paddy fields, 90 plants of each cultivar were cultivated. The cultivation process was as follows: in Kyoto, transplanting to paddy field on 19 June [20 days after sowing (DAS)], foliar spray treatment on 17 July (48 DAS), 29 July (60 DAS), 20 August (82 DAS) and 13 September (106 DAS), and harvest on 18 September (111 DAS) for Koshihikari and 8 October (131 DAS) for JRC-32 and JRC-33; in Okayama, transplanting to paddy field on 11 July (13 DAS), foliar spray treatment on 22 August (55 DAS) and 19 September (83 DAS), and harvest on 22 October (116 DAS). Rice plants (18 plants in Kyoto and 36 plants in Okayama of each cultivar) were harvested and dried in natural air, then evaluated.

For foliar spray treatment, the PPFM mixture (strains RS1, Rst and Sh) suspended in a solution containing spreading agent (0.02% polyoxyethylene nonylphenyl ether and 0.1% carboxymethyl cellulose) at a final OD_{600} = 1 was used. This PPFM mixture was sprayed on the growing rice plants (*ca*. 3 ml per plant), while the solution without PPFM was sprayed as a control.

Paddy field trials in a commercial paddy field

The effect of the PPFM treatment on crop yield was tested in commercial paddy fields. *O. sativa* cultivar Hakutsurunishiki was grown in a paddy field at Higashiyasuda, Taka, Hyogo Prefecture (35°02'20"N 134°57'30"E) from 2014 to 2018. Planting densities were 19, 18, 18, 14 and 14 hills m^{-2} in 2014, 2015, 2016, 2017 and 2018 respectively. The PPFM mixture containing strains RS1, Rst and Sh, and strain Rst alone was used for foliar spray treatment. In 2015, seed inoculation was conducted as described above with a mixture of strains By, RS1 and Sh.

Autoclave-killed cells and the cell wall polysaccharide fraction of strain Rst were also used for foliar spray treatment. Autoclave-killed cells were prepared from methanol-grown cells, harvested, washed twice with sterilized water and suspended in sterilized water. The cell suspension was autoclaved at 121°C for 20 min and diluted in a solution containing spreading agent to a final OD_{600} = 0.5.

The cell wall polysaccharide fraction was prepared from methanol-grown cells, harvested, washed twice with 50 mM NaCl and suspended in 50 mM EDTA and 2% SDS. After incubation at 37°C for 1 h, one-tenth volume of 3 M sodium acetate and 30% volume of ethanol were added to the suspension and the resultant suspension was kept at −20°C for overnight. The suspension was subjected to centrifugation at 8000 *g* for 5 min and the pellet was washed with 70% ethanol and suspended in a solution containing spreading agent.

In 2014, foliar spray treatment of the rice seedlings was done on 29 May (13 DAS), and after transplantation of the seedlings to the paddy field on 20 June (35 DAS), foliar spray treatments were done on 14 July (59 DAS), 5 August (81 DAS) and 8 September (115 DAS). In 2015, seed inoculation was done on 19 May and seeds were sown on 20 May. After transplantation to the paddy field on 16 June (27 DAS), foliar spray treatments were done on 7 August (79 DAS) and on 14 September (117 DAS). In subsequent years, foliar spray treatments were done before and/or after the heading date, in 2016 on 8 August (81 DAS) and 16 September (120 DAS), in 2017 on 8th August (80 DAS) and 6th September (109 DAS) and in 2018 on 9 August (81 DAS) and 14 September (117 DAS). Approximately 100 hills of rice plants were harvested from each test area (*ca*. 5–8 m^2) on 27 October 2014 (164 DAS), 22 October 2015 (155 DAS), 25 October 2016 (159 DAS), 6 November 2017 (170 DAS)

and 5 November 2018 (169 DAS), dried in natural air, and the weight of brown rice was measured. In the 2018 trial, number of panicles per hill was measured for 20 hills in each test area, and number of grains, rate of ripening and 1000 g weight were measured for two hills whose number of panicles was near the average value in each test area. Grains that sank in water of density 1.06 (60 g l^{-1} NaCl) were regarded as ripened grains.

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Conflict of interest

A.O., T.Y., T.A., H.Y. and Y.S are listed as inventors for Japan patent application no. 2018-078786 describing the method for foliar spraying of *Methylobacterium* spp. to increase crop yield. The other authors declare no competing interests.

Author contributions

H.Y. and H.I. designed research, performed experiments, analysed data and wrote the paper. D.T.D.T. isolated and characterized PPFM strains. A.T. and Y.O. designed and conducted paddy field experiments in Okayama and Kyoto, respectively, and edited the paper. A.O., T.Y. and T.A. designed and conducted paddy field experiments in Hyogo. Y.S. designed research, analysed data and wrote the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Evaluation of rice production in the paddy fields.

Fig. S2. Leaf greenization of rice seedlings in response to PPFM treatment.

Table S1. Biochemical characterization of PPFM isolates.

Table S2. Effect of treatment with the PPFM mixture on various rice cultivars.