

Effects of a supernatant from *Aspergillus oryzae* culture on the regulation of crassulacean acid metabolism (CAM) and pinitol biosynthesis in the common ice plant, *Mesembryanthemum crystallinum* L.

Shigeki SAITO^{1, 2)}, Kotaro YAMANO²⁾, Haruki UEDA²⁾, Yasushi TANI^{1, 2)},
Makoto HAYAKAWA³⁾, Akihisa TSUJI³⁾, and Hisaaki MIHARA^{2) †}

¹⁾ *Ritsumeikan Global Innovation Research Organization, Ritsumeikan University**,

²⁾ *Department of Bioengineering, College of Life Sciences, Ritsumeikan University***,

³⁾ *Nihon Advanced Agri Corporation****

Summary

A facultative halophyte *Mesembryanthemum crystallinum* L. changes its carbon fixation mechanism from C3 to crassulacean acid metabolism (CAM) in response to environmental stress such as drought and salinity. Induction of CAM also results in the accumulation of metabolites like malate, betacyanin, proline, and pinitol in the plant with mechanisms that are unknown as yet. In order to explore the factors that lead to the formation of these metabolites in this plant, we treated *M. crystallinum* L. with culture supernatants of *Aspergillus oryzae*, *Nectria gracilipes*, and *Rhizopus* sp. Among the fungi tested, culture supernatant from *A. oryzae* induced typical CAM phenotypes in this halophyte species even in the absence of salt. Endogenous malate levels in the treated plants increased 5-fold in comparison with those in the control plants. We investigated the expression patterns of genes like CAM-specific *Ppck* and *Ppc1*. Our analysis of expression patterns of these genes showed no significant increase in their expression levels in the *A. oryzae*-treated plant, indicating that the accumulation of malate was independent of CAM-photosynthesis. When the plant was treated simultaneously with the culture supernatant and 400 mM NaCl, circadian regulation of *Ppck* was disturbed. Expression patterns of a pinitol biosynthesis gene (*Imt*) in the treated plants also showed anomalies in the circadian rhythm. On the basis of these results, we suggest that *Ppck* and *Imt* genes may be regulated by a novel mechanism independent of CAM.

Introduction

A facultative halophyte *Mesembryanthemum crystallinum* L., also known as the common ice plant, has been attracting the attention of plant scientists for decades because of its unique carbon fixation strategy and high tolerance towards salinity. The plant uses the C3 carbon fixation system under normal growth conditions, however, photosynthesis by the crassulacean acid metabolism (CAM) is induced in response to environmental stress such as high salinity, drought, temperature changes, and high irradiance^{1–6)}. Induction of CAM is also seen in some developmental stages of the plant. When the carbon fixation metabolism is shifted to CAM, CO₂ is fixed by phosphoenolpyruvate carboxylase (PPC) during the nocturnal period, resulting in the formation of oxaloacetate, which is subsequently converted to

malate to be stored in vacuoles. As the period of light commences, the plant closes its stomata to avoid dehydration and removes CO₂ from the malate in the cytosol, producing pyruvate as a byproduct. The released CO₂ is then re-fixed by Rubisco in the Calvin cycle and is incorporated into carbohydrates^{4–6)}. These events follow the circadian rhythm^{5,7)}. The induction of CAM also manifests as a range of morphological characteristics⁸⁾. A dwarf plant with dark green leaves is recognized as a typical CAM phenotype. In response to environmental stress, the plant is covered with bag-shaped “bladder cells,” where the excess absorbed salt is accumulated. Betacyanin, an indole-derived pigment found in some plants, is also stored in these bladder cells⁹⁾.

Environmental stress factors that induce CAM also lead to the biosynthesis of pinitol (1-D-3-O-methyl *chiro*-inositol)^{10–12)}. Pinitol is a well-known compatible solute for plants, just like

*** Address : 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan

*** Address : 1281-8 Tamura-cho, Nagahama, Shiga 526-0829, Japan

† To whom correspondence should be addressed. Tel: +81 77 561 2732; Fax: +81 77 561 2659;

E-mail address: mihara@fc.ritsumei.ac.jp (H. Mihara).

proline, glycine betaine, and sugars¹³). *M. crystallinum* L. showed the accumulation of both pinitol and proline in response to osmotic stress because of drought and high salinity^{14,15}. Pinitol is synthesized from *myo*-inositol in 2 steps¹⁶⁻¹⁸. The biosynthesis starts with the transfer of the methyl group of *S*-adenosyl-*L*-methionine to *myo*-inositol by the inositol 4-methyl transferase (IMT). The resultant ononitol (1-*D*-4-*O*-methyl *myo*-inositol) is then converted to pinitol by an epimerase.

The demand for pinitol in the pharmacological industry is increasing because of its insulin-like effect¹⁵. Pinitol was first isolated from the leaves of *Bougainvillea spectabilis*, which were used as a traditional treatment for diabetes in Asia and the West Indies^{19,20}. Narayanan *et al.* reported that administration of pinitol reduced the blood sugar level in alloxan-induced diabetic albino mice¹⁹. Since pinitol has no anti-diabetic effect on its own, it is proposed that pinitol is demethylated in mammalian cells by an enzyme, and the resulting *chiro*-inositol serves as an insulin mediator to decrease plasma glucose concentration²¹.

Plant-derived pinitol extracts are useful as nutritional supplements. Pinitol has mainly been obtained from the pods of the carob tree (*Ceratonia siliqua* L.) and soybean (*Glycine max* L.)^{15,22}. In *M. crystallinum* L., pinitol forms 71 % of its soluble carbohydrate fraction and 9.7 % dry weight in the long-term stressed leaves¹⁴. Since *M. crystallinum* L. is edible and popular as a vegetable in some countries, it can be an ideal source of pinitol for the production of a nutritional supplement. However, to produce pinitol, the plant is usually treated with a high concentration of NaCl. Since the plant develops bladder cells on its surface and stores excess amounts of NaCl in the cells, the extracts also contain high amounts of NaCl in addition to pinitol. These high levels of salt are not suitable for using extracts of the plant as nutritional supplements. Therefore, elucidating the mechanisms of induction of the IMT gene (*Imt*) without NaCl treatment is important in order to use this plant as a nutritional supplement.

It is well known that treatment of plants with biological compounds such as chitin oligosaccharides and flagellin, which work as elicitors, causes an array of defensive reactions, leading to the accumulation of plant secondary metabolites²³. Therefore, we thought that it is possible to enhance pinitol production by treating the plant with secreted products or cell extracts from microbes. Filamentous fungi are chosen particularly for this purpose, since culture supernatant of these fungi contain a wide range of peptides and enzymes as well as bioactive small chemical compounds such as penicillins and cephalosporins²⁴. Furthermore their ease of cultivation and high productivity of se-

creted products make industrial production of these elicitors feasible.

In this study, we have investigated the effects of culture supernatants from filamentous fungi on the regulation of CAM and pinitol biosynthesis. Culture supernatants from *Aspergillus oryzae*, *Nectria gracilipes* and *Rhizopus* sp. were added to the growth medium of the ice plant and their effects on the plant's phenotypes were examined. The correlation between accumulation of malate and the expression levels of phosphoenolpyruvate carboxylase kinase gene (*Ppck*), phosphoenolpyruvate carboxylase gene (*Ppc*) and inositol 4-methyl transferase gene were also analyzed.

Materials and Methods

Chemicals and fungi

All the chemicals and reagents used in this study were purchased from Nacalai Tesque Inc. (Kyoto, Japan), Sigma-Aldrich Inc. (St. Louis, USA) or Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless specified otherwise. *A. oryzae* RIB 40 and *N. gracilipes* JCM 7938 were obtained from the National Research Institute of Brewing (Higashi-hiroshima, Japan) and RIKEN BioResource Center (Tsukuba, Japan), respectively. *Rhizopus* sp. was a kind gift from Prof. Mamoru Wakayama at Ritsumeikan University (Kusatsu, Japan).

Plant growth conditions

Seeds of *M. crystallinum* L. were purchased from Nihon Advanced Agri Corporation (Nagahama, Japan). For germination, the seeds were surface-sterilized using a sterile solution (0.3 % sodium hypochlorite, and 0.05 % Tween 20) and germinated on Murashige and Skoog (MS) plates (0.43 % Murashige and Skoog salts, 1 x B5 vitamins, 2 % sucrose, and 0.8 % agar, pH 5.8). After 1 week, the germinated seedlings were transferred to another batch of MS medium in plant tissue culture containers (6 plants/container), and incubated for 3 weeks. The plants were moved to MS media containing 400 mM NaCl, 5 % (v/v) fungal culture supernatant, or 5 % YPG (v/v) medium as the control (4 plants/container). The 4th leaves from the apical leaf were collected after 3-4 weeks of incubation. The plants were grown in a growth chamber at 24 °C under photosynthetic flux of 100-120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ of white fluorescent light on a 16-h light/8-h dark photoperiod.

Preparation of MS media containing the culture supernatants from filamentous fungi

Filamentous fungi were grown on YPG plates (0.5 % yeast extract, 0.5 % peptone, 1 % glucose, 0.5 % NaCl, and

1 % agar) until spores formed. A 1-cm² piece of the spore-formed fungus was cut in pieces, suspended in 100 mL of YPG medium, and incubated in a 500 mL Sakaguchi flask on a rotary shaker at 70 rpm at 30 °C for 8 days. The culture was filtered through a filter paper, and the filtrate was further filter-sterilized using a 0.45- μ m syringe filter (Sartorius Stedim Biotech, Goettingen Germany). Freshly filter-sterilized culture supernatants were added to autoclaved MS medium to the final concentration of 5 % (v/v). Plants were transferred to the media within 2 days.

Determination of malate

Malate was extracted from leaves as described by Koreeda *et al.*²⁵⁾. The leaf tissues (ca. 100 mg) were collected at the beginning and the end of the light period and stored at -80 °C until further analysis. The samples were ground in 340 μ L of ice-chilled 6 % (w/v) perchloric acid. The homogenates were subjected to centrifugation (10,000 rpm, 10 min, 4 °C) to remove the cell debris. The supernatants were adjusted to pH 9 with approximately 60 μ L of 5 M KOH, and the precipitates were removed by centrifugation (10,000 rpm, 10 min, 4 °C). Concentrations of malate in the leaf extracts were determined enzymatically by the method of Nunes-Nesi *et al.*²⁶⁾. We added 100 μ L of the extracts to 890 μ L of the reaction mixture containing 100 mM Tricine/KOH at pH 9, 3 mM NAD⁺, 1 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 0.4 mM 5-methylphenazinium methyl sulfate (PMS), and 0.5 % (v/v) Triton X-100. The reaction mixture was pre-incubated for 5 min at 30 °C. After measuring the absorbance at 570 nm for 1 min, 10 μ L of 1 U malate dehydrogenase was added, and the absorbance was measured until it reached a plateau.

Semi-quantitative RT-PCR

The leaf tissues (ca. 100 mg) were collected at the beginning and the middle of the light period, immediately frozen in liquid nitrogen, and stored in -80 °C for subsequent analysis. Total RNA samples were extracted with Sepazol (Nacalai Tesque Inc., Kyoto Japan), according to the manufacturer's instructions. The RNA extracts were treated with RNase-free DNaseI (Takara Bio Inc., Shiga, Japan) to eliminate genomic DNA contamination. The DNase I-treated RNA was diluted to 50 ng/ μ L, and 1 μ L of it was used for the following RT-PCR reaction. Single-strand cDNAs were synthesized using RiverTraAce- α - (Toyobo Co., Ltd., Osaka, Japan) using random primers. Each 10 μ L of PCR reaction contained 1 μ L of the RT reaction mixture, and PCR reaction was carried out under the following conditions using KOD-Plus-Neo (Toyobo Co., Ltd., Osaka, Japan) at 94 °C for 2 min (1 cycle); 98 °C for 10 s, 57 °C for 30 s, 68 °C for 30 s (24, 28 or 32 cycles). The

primers used in this study were as follows: McPpcK_fwd, 5'-GTGTGAGAGCTTCAAGAGAGAATACCAG-3'; McPpcK_rev, 5'-CTTGACATCATCTTCCTCAACAAATCCC-3'; McPpc1_fwd, 5'-GGGGTGAAGCTCACAATGTTTCATGGAC-3'; McPpc1_rev, 5'-CTCTAGCAGGTCTTTATGCCAGCG-3'; McImt_Fwd, 5'-TTCTCAAAGGTGTATGGTCCAGCTCC-3'; McImt_Rev, 5'-ACAAGGATGATCTTCCCTCCCTTTGCC-3'; McFnr_Fwd, 5'-ATTGCCAGCAGGCCCTTGGGC-3'; McFnr_Rev, 5'-TCGAACCAGTCAATACCATCTTCTGCG-3'. The PCR primers were designed to span an intron to distinguish cDNA-derived PCR products from the genomic DNA. PCR products were analyzed by performing 1 % agarose gel electrophoresis. Densitometric analysis of ethidium bromide-stained PCR products was performed using the ImageJ program (developed at the National Institute of Health and available on the internet at <http://rsbweb.nih.gov/ij/>). Sequences used in this article can be found in the GeneBank under accession numbers AF158091 (Ppck), X13660 (Ppc1), M87340 (Imt), and M25528 (Fnr).

Results

Effects of culture supernatants from filamentous fungi on the phenotype of *M. crystallinum* L.

In order to regulate CAM-specific genes or pinitol biosynthesis genes in the absence of NaCl, we treated *M. crystallinum* L. with culture supernatants from filamentous fungi as well as chemicals including plant hormones and intermediates in the CAM pathway. Among the fungi tested, the culture supernatants from *A. oryzae* showed significant effects on the growth of *M. crystallinum* L. (Fig. 1a). The plants treated with the culture supernatants in the absence of NaCl showed a dwarf phenotype with apparent accumulation of betacyanin indicating induction of CAM under these conditions. On the other hand, plants treated with either *N. gracilipes* or *Rhizopus* sp. did not show significant differences in comparison with the control plants. Plant hormones and the intermediates such as oxaloacetate and pyruvate did not cause a severe CAM phenotype either (data not shown).

To examine whether CAM was induced in the *A. oryzae*-treated plants, the endogenous concentrations of malate were measured. The endogenous levels of malate were at least 5 times higher in the *A. oryzae*-treated plants than those in the control plants (Fig. 2). As we expected, the endogenous levels of malate increased at dawn and decreased at dusk, suggesting that the carbon fixation mechanism was shifted to CAM. Although the *Rhizopus*-treated plant did not show a significant change in the phenotype (Fig. 1a), it accumulated rather higher levels of malate (Fig. 2). Inter-

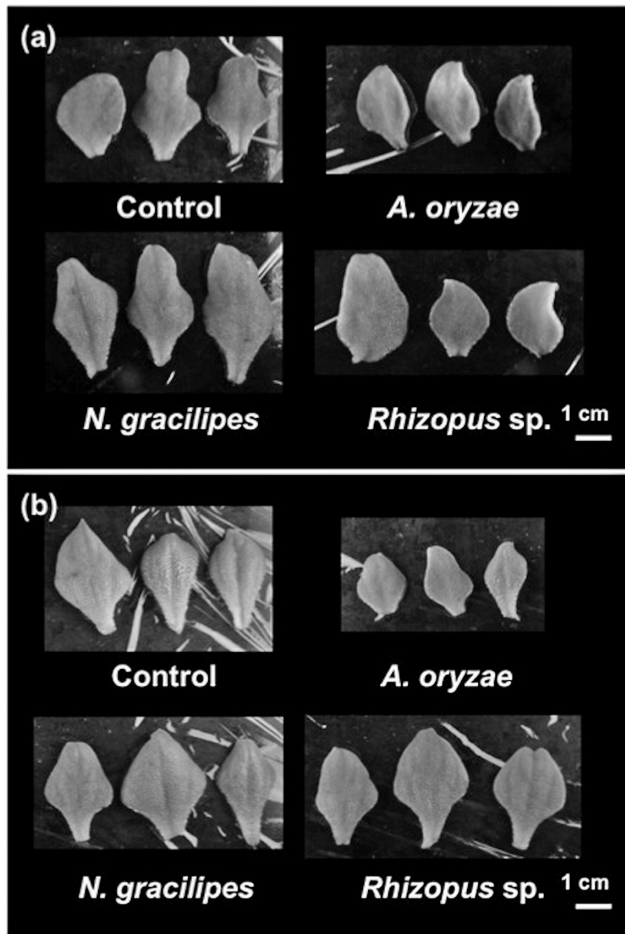


Fig. 1 Effects of culture supernatants from filamentous fungi on *Mesembryanthemum crystallinum* L. Four-week-old *M. crystallinum* L. were grown for another 4 weeks on MS medium containing 5% (v/v) fungal culture supernatant prepared from either *Aspergillus oryzae*, *Nectria gracilipes*, or *Rhizopus* sp., or 5% (v/v) YPG medium as a control. Leaf samples were collected from each plant grown in the absence (a) or presence (b) of 400 mM NaCl.

estingly, the endogenous levels of malate in this plant at dusk were higher than that at dawn. Treatment with culture supernatant from *N. gracilipes* showed no significant effect on the levels of malate in the plants (Fig. 2).

We also examined whether the incubation conditions of *A. oryzae* affected the phenotype. The culture supernatant of *A. oryzae* prepared from a culture with a rotation of 70 rpm caused a severe CAM phenotype. On the other hand, supernatant isolated from a culture grown at 100 rpm showed a normal phenotype (data not shown). The pH of the YPD medium incubated with *A. oryzae* for 8 days was 8.6 for a 70 rpm culture and 8.9 for 100 rpm culture. The MS medium containing 5% (v/v) of the culture supernatant from *A. oryzae* showed a pH of 6.7 and 6.8. This suggests that the pH in the medium does not affect the phenotype, but the secreted product(s) in the supernatant are responsible for the shift in the CAM phenotype. The culture supernatant prepared from the 70 rpm culture was used for the subsequent experiments.

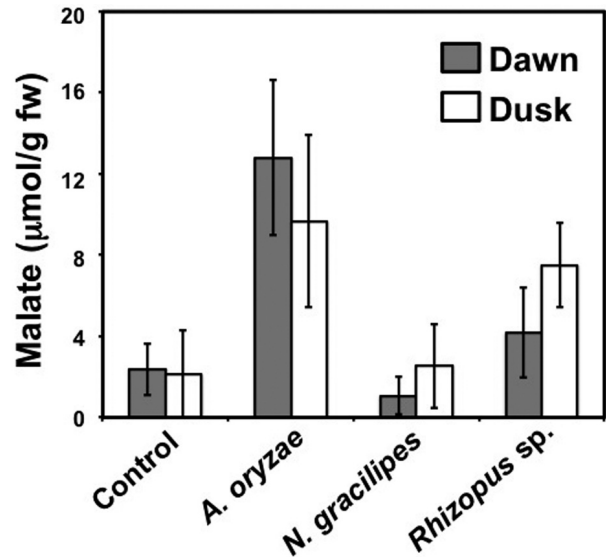


Fig. 2 Diurnal changes in the endogenous levels of malate induced by culture supernatants from filamentous fungi. Four-week-old *M. crystallinum* L. were grown for another 4 weeks on MS medium containing 5% (v/v) fungal culture supernatant prepared from either *A. oryzae*, *N. gracilipes*, or *Rhizopus* sp., or 5% (v/v) YPG medium as a control in the absence of NaCl. Leaf samples were collected at the beginning (dawn) and at the end (dusk) of the light period. The data represent the mean values obtained from 3 independent experiments. Error bars indicate the SD.

Effects of the culture supernatant from *A. oryzae* on the expression levels of CAM-related genes

The expression patterns of CAM-specific *Ppck* and *PpcI* were investigated by performing semi-quantitative PCR (Fig. 3). Ferredoxin NAD⁺ reductase gene (*Fnr*) was used to normalize each of the PCR reactions, since expression levels of this gene remain constant in leaves from well-watered *M. crystallinum* L. as well as in leaves from the plants grown under saline conditions²⁷⁻²⁹. Both these genes were upregulated by 400 mM NaCl treatment, indicating the induction of CAM by salinity (Fig. 3b, c). Although the plants treated with the culture supernatant from *A. oryzae* in the absence of NaCl showed a severe dwarf phenotype, neither *Ppck* nor *PpcI* were induced. When *M. crystallinum* L. was treated with 400 mM NaCl, high expression levels of *Ppck* were detected during the day and night in the *A. oryzae*-treated plant (Fig. 3b). On the other hand, the expression of *PpcI* in the plant showed a pattern similar to that of the control plants with some enhancement (Fig. 3c).

The expression of the *Imt* gene was also investigated. This gene encodes a key enzyme required to produce pinitol, which helps to regulate osmotic pressure in the cell, and is transcriptionally induced in response to water stress in *M. crystallinum* L.¹⁰⁻¹². Addition of culture supernatant from *A. oryzae* did not cause any induction of *Imt* in the absence of NaCl. Expression of *Imt* was induced only when the plant was treated with NaCl (Fig. 3d). Although it

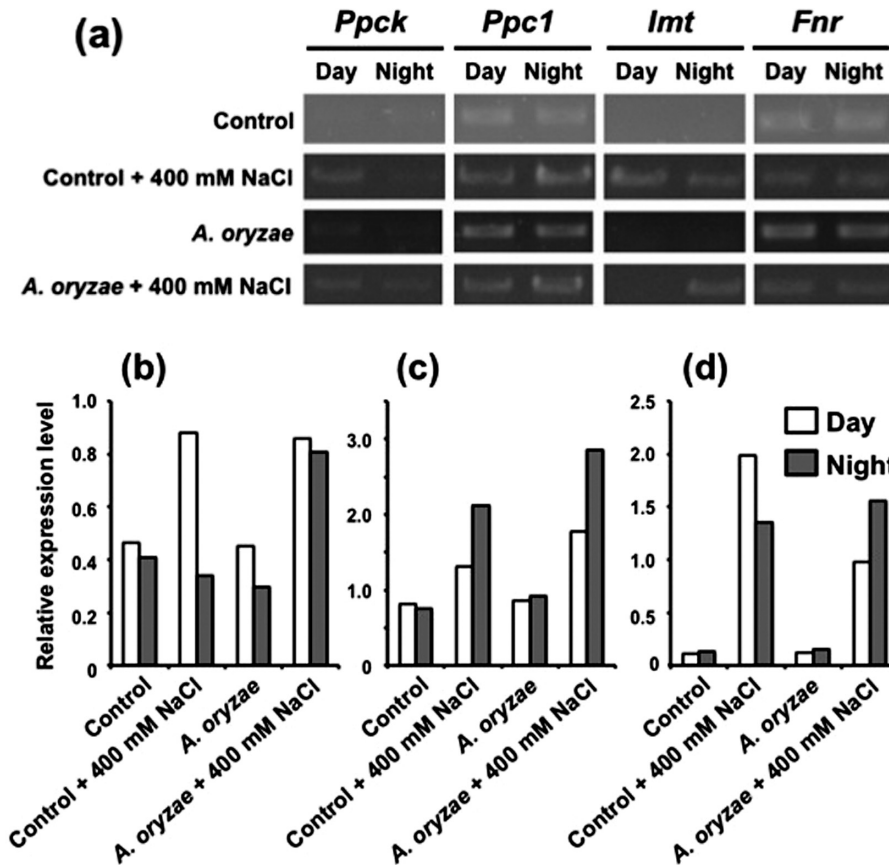


Fig. 3 Expression levels of the CAM-specific *Ppck* and *Ppc1* genes and the pinitol biosynthesis gene *Imt*, induced by the culture supernatant from *A. oryzae*. Samples of leaves were collected at the middle (day) and the end (night) of the light period. Semi-quantitative RT-PCR analysis of each of the gene transcripts was carried out using gene specific primers (a). The densitometry data for the band intensities of *Ppck* (b), *Ppc1* (c) and *Imt* (d) were generated from the gel images shown in the panel (a) with the ImageJ program. The expression of *Fnr* was used as a control to normalize the expression levels of each gene transcript. The data represent the mean values obtained from at least 2 independent experiments.

showed higher expression levels during the day in the control plants, induction of this gene at night was observed in the *A. oryzae*-treated plant.

Discussion

In this study, we investigated the effects of culture supernatants from filamentous fungi on the CAM-related metabolism in *M. crystallinum* L. Since the plant accumulates pinitol, which has an insulin-like effect on the plasma glucose level, daily consumption of the plant or its extracts would be a promising option for a patient who suffers from diabetes.

CAM is characterized by nocturnal uptake and assimilation of CO₂ into malate. This regulation is achieved by an internal circadian rhythm that controls the expression and activity of PPCK, which in turn regulates the phosphorylation state of PPC^{29, 30}. In keeping with our expectations, the accumulation of malate at dawn and its decrease at dusk was observed in the plants treated with the culture super-

natant of *A. oryzae* (Fig. 2). However, the expression patterns of *Ppck* and *Ppc1* did not change significantly between the treated plants and the control ones (Fig. 3b, c). On the other hand, when *M. crystallinum* L. was exposed to high salinity, the culture supernatant from *A. oryzae* induced the expression of *Ppck* in the nocturnal period (Fig. 3b). Although the gene controls the phosphorylation state of PPC, the nocturnal expression did not cause any effect on the expression of *Ppc1*. The expression of *Ppc1* was slightly enhanced by the *A. oryzae* treatment and yet it followed the circadian rhythm. It is well known that the expression of *Ppck* and *Ppc1* follow the circadian rhythm as we observed in the control plants treated with 400 mM NaCl. The disruption of the circadian expression pattern of *Ppck* in the *A. oryzae*-treated plants implies that the culture supernatant induced another signaling pathway that can regulate *Ppck*.

It is thought that plants synthesize pinitol as a compatible solute in response to osmotic stress¹³. The positive correlation of the accumulation of pinitol and other cyclitols

such as *myo*-inositol and *chiro*-inositol in response to the concentration of salt was observed in various plants species such as *Honkenya peploides*³¹, *Sesbania bispinosa*³², and wild rice (*Porteresia coarctata*)¹⁸, chick pea (*Cicer arietinum*)³³, sea lavender (*Limonium perezii*)³⁴, and *M. crystallinum*¹⁵. In *M. crystallinum* L., biosynthesis of pinitol is transcriptionally regulated by exposure to NaCl³⁵. The amount of sodium uptake also depends on the concentration of *myo*-inositol³⁶. Borland *et al.* have suggested that pinitol would also work as a scavenger to remove reactive oxygen species (ROS)³⁷. Plants produce ROS such as O₂^{-•}, H₂O₂ and OH• in response to various abiotic factors. The generated ROS work as signaling molecules capable of migrating over short and long distances in plants, and induce the expression of various stress-related genes^{38, 39}. However, because of their high reactivity, excessive accumulation of intracellular ROS would cause membrane damage as well as degradation of nucleic acids and proteins. Therefore, we expected that if treatment with the culture supernatant of *A. oryzae* led to an increase in the ROS present in the cells of the ice plants, biosynthesis of pinitol would be enhanced. The *Imt* gene, however, did not respond to the treatment with culture supernatant from *A. oryzae* in the absence of NaCl. Treatment with culture supernatant from *A. oryzae* altered the expression of *Imt* only when the plants were simultaneously exposed to high salinity. The higher expression level of *Imt* during the night might be because of the effect of culture supernatant of *A. oryzae* on the mechanism of regulation of *Imt* expression.

In conclusion, *M. crystallinum* L. treated with the culture supernatant of *A. oryzae* showed a CAM-like phenotype. Although accumulation of malate at dawn indicated the shift of the carbon fixation mechanism from C3 to CAM, the treated plants did not show typical expression patterns of CAM-specific genes such as *Ppck* and *Ppc1*. Specifically, the expression of *Ppck* in the *A. oryzae*-treated plants was aberrant in that it did not follow the circadian rhythm, indicating the existence of a CAM-independent mechanism for the regulation of *Ppck* expression. The culture supernatant also influenced the expression patterns of *Imt*. Although the gene was induced only when the plant was treated with high concentration of NaCl, our results suggest the possibility of CAM-independent induction of *Imt*. The combination of other abiotic stresses with the culture supernatant might make the induction of the gene possible without salinity.

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