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Transiently Expressed Salt-Stress Protection of Rice by Transfer of a Bacterial Gene, *mtlD*

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Abstract Productivity of a rice plant is greatly influenced by salt stress. One of the ways to achieve tolerance to salinity is to transfer genes encoding protective enzymes from other organisms, such as microorganisms. The bacterial gene, *mtlD*, which encodes mannitol-1-phosphate dehydrogenase (Mtl-DH), was introduced to the cytosol of a rice plant by an imbibition technique to overproduce mannitol. The germination and survival rate of the imbibed rice seeds were markedly increased by transferring the *mtlD* gene when it was delivered in either a *pBIN19* or *pBmin* binary vector. When a polymerase chain reaction was performed with the genomic DNAs of the imbibed rice leaves as a template and with *mtlD*-specific primers, several lines were shown to contain an exogenous *mtlD* DNA. However, a reverse transcription (RT)-PCR analysis revealed that not all of them showed an expression of this foreign gene. This paper demonstrates that the growth and germination of rice plants transiently transformed with the bacterial gene, *mtlD*, are enhanced and these enhancements may have resulted from the expression of the *mtlD* gene. The imbibition method employed in this study fulfills the requirements for testing the function of such a putative gene *in vivo* prior to the production of a stable transgenic plant.

Key words: *mtlD* (mannitol-1-phosphate dehydrogenase), salt tolerance, metabolic engineering, polyol, imbibition, transient expression

Plants are continuously exposed to environmental stress, thereby influencing their development, growth, and consequently productivity. High salinity stress severely limits the growth and agricultural productivity of crops. However, plants are able to adapt themselves to such environmental stress by balancing intracellular metabolites through osmotic stress perception and signaling [2]. To adjusting against salinity, osmolytes are accumulated either in the cytoplasm or vacuole to increase the osmotic pressure of the cell. For example, a high concentration of the protective metabolite polyol, trehalose, occurs in many anhydrobiotic organisms that survive a water deficit, although it is rare in higher plants [5]. To develop crop plants grown in high-salt soil, different strategies have been suggested, including engineering a salt-tolerant transgenic plant. Recently, it was reported that the constitutive production of bacterial mannitol-1-phosphate dehydrogenase (Mtl-DH: E.C.1.1.1.17) could confer osmotolerance in transgenic *Arabidopsis* [12] as well as in tobacco plants [10, 11]. Mtl-DH encoded by the *mtlD* gene catalyzes the production of acyclic polyol, mannitol, and can accumulate this polyol in transgenic plants. When *mtlD* was expressed in transgenic plant, it reduced fructose-6-phosphate to mannitol-1-phosphate, and subsequently mannitol-1-phosphate was dephosphorylated to form mannitol [10]. The resulting improved salt tolerance is attributed more to the osmoprotective properties of mannitol than to osmotic adjustment. One other possible mechanism by which plants can survive salt stress is to compartmentalize sodium ions away from the cytosol. In most saline soils, Na^+ is the major toxic cation because Na^+ disrupts K^+ nutrition [6]. The overexpression of a vacuolar Na^+/H^+ antiport in transgenic *Arabidopsis* plants promotes sustained growth and development in a saline environment [1].

Although many putative salt-tolerant genes have been identified, it is difficult to investigate whether a gene transfer can improve salt tolerance in plants, since there are many obstacles to foreign gene expression in higher plants [4]. Accordingly, before attempting to prepare a transgenic plant, a simpler method should be applied to test the effect of these putative salt-tolerant genes.

In this study, the bacterial *mtlD* gene encoding Mtl-DH was transferred into rice embryos using an imbibition technique to examine the putative osmoprotectant Mtl-DH in the rice, because the function of this gene in either monocotyl plants or crop plants has not yet been proved. It was demonstrated that the exogenous Mtl-DH functions in producing sugar alcohol and the accumulation of this polyol produced a salt-tolerance in the rice plant.

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The bacterial gene *mtlD*, engineered in *pBIN19*, was given by Dr. Hans J. Bohnert as a gift. The 2.3 kb *mtlD* fused to a 2x35S promoter fragment was transferred either in *pGEM-4z* (Promega) or in *pBmin* (a gift of Dr. I-H. Hwang) (Fig. 1). The imbibition technique was performed as mentioned previouly [8]: Fifty dry rice dissected embryos were imbibed in a solution containing 100 μg/ml of plasmid DNA in SSC (15 mM NaCl, 1.5 mM Na-citrate) and 20% DMSO for 12 h at 4°C, and then sterilized. The imbibed embryos were germinated and grown on agar-solidified MS media. The fifty imbibed embryos were then individually placed on petri dishes and incubated in a growth chamber (16 h light, 150-200 μmol m^{-2} s^{-1} photon flux density). The germination rate was determined by the number of seedlings that were germinated after 3 days of incubation on the MS media containing either 0% or 1.3% (w/v) NaCl. For testing the salt-tolerance, the number of plants that survived on the MS agar media containing 1-1.3% NaCl were counted at 1, 2, 3, and 4 weeks after germination. A leaf from the imbibed plants was harvested and the genomic DNA was extracted according to the manufacturer's manual (Qiagen). A fragment containing CaMV35S 5' and the bacterial *mtlD* gene was amplified by PCR using the leaf genomic DNA as a template. The gene-specific primers used were the CaMVII primer (5'-TCATTTCATTTGGAGAGGAC-3') and the *mtlD*-D3 primer (5'-CTTTCTTCCATCGCACCTTTTACCA-3'). The amplified products were analyzed on a 1.5% agarose gel. The total RNA was extracted from a plant containing the exogenous *mtlD* gene. A reverse transcription-PCR was performed according to the manufacturer's manual (One-step RT-PCR, TAKARA). *mtlD* gene-specific primers were used for the PCR (*mtlD*-U1 primer, 5'-GAGCAGGTAGAT-ACCGTTTCC-3'; *mtlD*-D1 primer, 5'-CCCGTATTCAGGG-TGAAGAG-3'). The PCR products were electrophoresised and blotted on a nylon membrane (Hybond-N). The blotted membrane was then hybridized with a ^{32}P-labeled probe. The blot was exposed to x-ray film to visualize the signals.

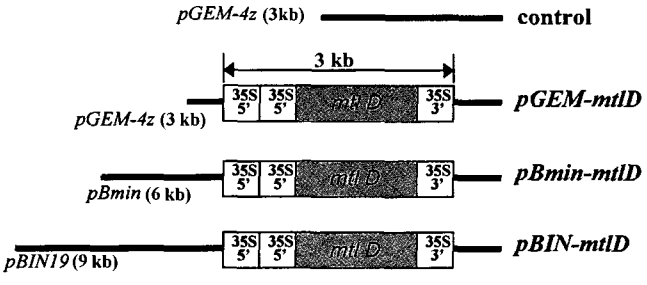

Fig. 1. Schematic diagram of plasmid constructs containing the *mtlD* gene.

The CaMV 35S promoter sequence (CaMV 5') and terminator sequence (CaMV 3') were used to express the bacterial gene, *mtlD*. The constructs, *pBIN-mtlD* and *pBmin*, were provided as gifts from H. J. Bohnert and I.-H. Hwang, respectively. The constructs, *pBmin-mtlD* and *pGEM-mtlD*, were constructed by transferring the expression cassette (CaMV 5'::*mtlD*:: CaMV 3') from *pBIN-mtlD*.

For transferring the exogeneous gene into plants using the imbibition technique, three different plasmid DNAs were used to make the recombinant *mtlD* constructs (Fig. 1). After the rice embryos imbibed with the *mtlD* gene constructs were fused with the double CaMV 35S promoters, the imbibed embryos were germinated and grown on high salt concentration MS agar plates for up to 4 weeks. To determine the ability to germinate under salt-stress, the number of germinated embryos was counted after three days of incubation and compared with that in the control. Figure 2 shows that the germination rate was slightly enhanced by the transfer of the constructs containing the *mtlD* gene. This result supports those of other reports which showed that *mtlD*-expressing *Arabidopsis* seeds had

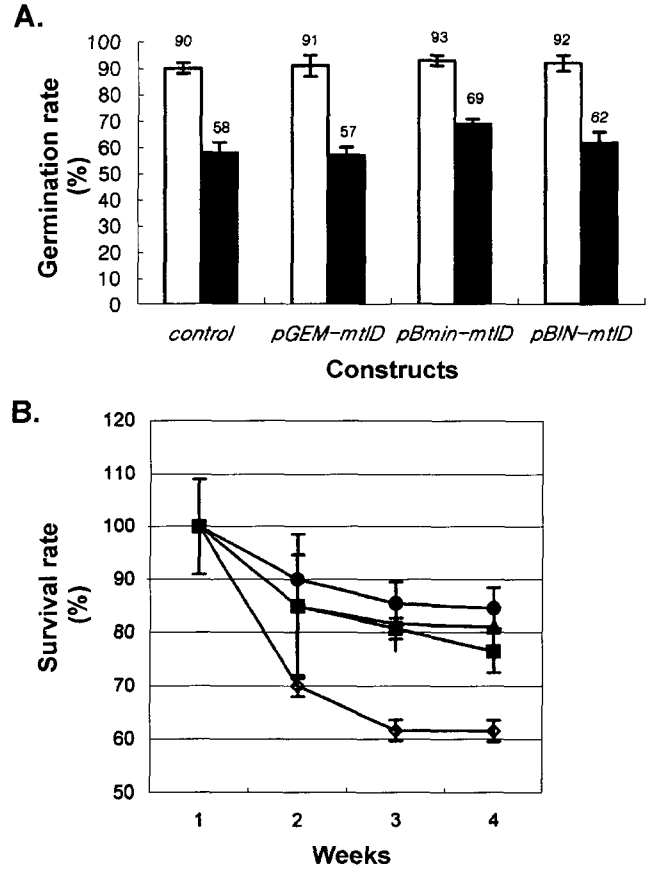

Fig. 2. Effect of imbibition with *mtlD* gene on the germination and growth rate of imbibed rice embryos on media containing NaCl (1.3% w/v).

Three different *mtlD*-containing constructs and one control (*pGEM-4z*) were used for testing the efficiency of the exogenous gene expression in the rice system; control (*pGEM-4z*). (A) Germination rate (open bar, 0% NaCl media; filled bar, 1.3% NaCl media) and (B) survival rate was determined as the relative number of germinated seeds to the 50 imbibed rice seeds, and as the relative number of surviving plants to the total number of germinated embryos, respectively. The number of surviving rice plants was observed at 2, 3, and 4 weeks after germination on a MS agar plate in control (open diamond, *pGEM-4z*), *pGEM-mtlD* (filled square), *pBmin-mtlD* (filled triangle), *and pBIN-mtlD* (filled circle). This experiment was repeated three times.

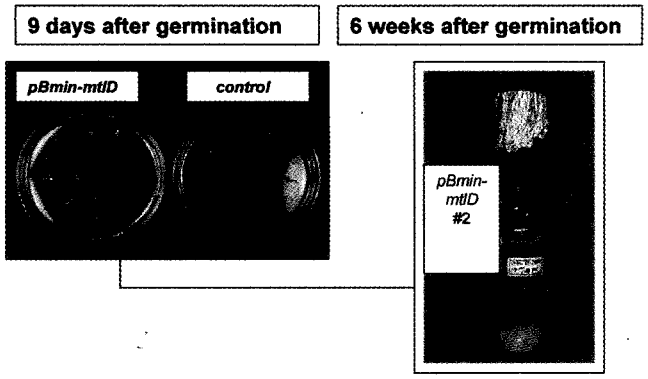

Fig. 3. Rice plants imbibed with the *mtlD*-containing construct or with control DNA.
Left panel; the imbibed rice plants with *pBmin-mtlD* (**pBmin-mtlD**) or *pGEM-4z* (**control**) 9 days after germination. Right panel; the line #2 imbibed rice plant was transferred into an individual medium containing NaCl (1.3%) and cultured up to 6 weeks.

the ability to germinate in high salt conditions [12]. There were some plants that escaped from the high salt environment without a foreign gene transfer (control in left panel of Fig. 3). However, they were unable to grow much longer and died within 6 weeks. Only a few rice plants, such as rice line #2, maintained any further growth (Fig. 3). Although a binary vector was not necessary for the introduction of a foreign gene into a rice plant by imbibition, it resulted in a higher survival rate of the rice on high salt media than a simple plasmid vector, such as *pGEM* (Fig. 2). When the *mtlD* gene in *pBmin* was delivered into and expressed in the rice system, it exhibited the highest germination and survival rates. The only difference between *pBmin* (6 kb) and *pBIN19* (10 kb) is their molecular size. Both vectors were developed as binary vectors. Approximately 40% of the 1-week old control seedlings died within 2–3 weeks of incubation on the high salt media. However, the imbibed plants showed a higher survival rate even up to 4 weeks after germination. Although their exact function in plants is still unclear, several studies suggest that osmolyte, like mannitol, may protect the plant from salinity stress [11, 12]. A relationship between sodium and metabolite concentrations has been reported for a number of halotolerant species and also for salt-adapted cell-suspension cultures [3]. These observations support the feasibility of a strategy for engineering osmolyte synthesis and accumulation. It is unlikely that the bigger molecular size of this construct would be less efficient for physical gene introduction methods, such as imbibition or electroporation (Fig. 2). To examine the *mtlD* gene transfer in rice plants, PCR was performed using the genomic DNA of such salt-tolerant plants as a template, and CaMVII and *mtlD*-D3 as gene primers. The PCR-products were eletrophoresised on an agarose gel as shown in Fig. 4A. A RT-PCR analysis was then performed using gene-specific primers (*mtlD*-D1 and *mtlD*-U1). Two plants among the three that showed the

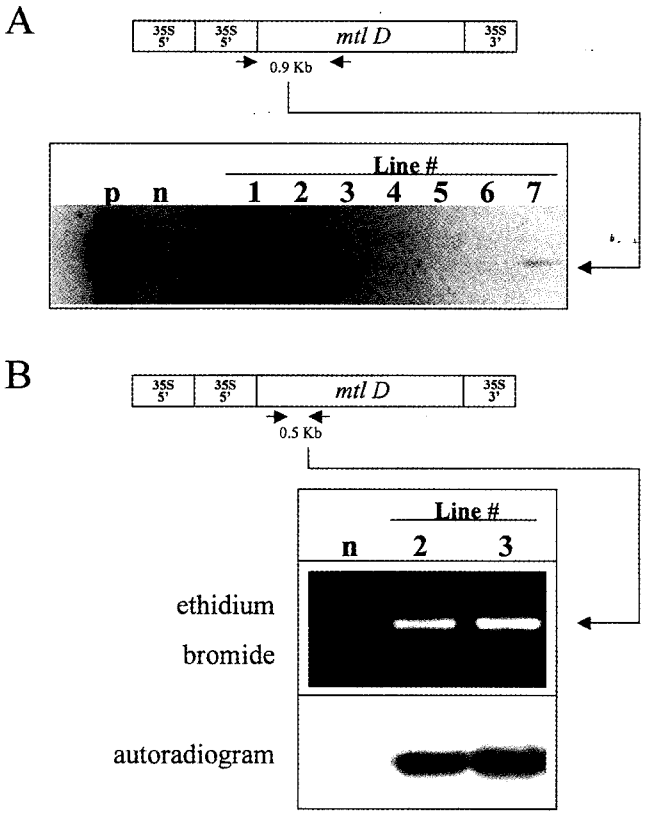

Fig. 4. Identification of an *mtlD* gene transfer (A) and expression (B) in the imbibed rice plants using PCR.
A. The genomic DNAs of the rice plants were used as the template and the CaMVII and *mtlD*-D3 primers were used as the foreign gene-specific primers for the PCR amplification. The PCR products are shown on a 1.5% ethidium bromide-stained agarose gel after electrophoresis. B. RT-PCR analysis of the *mtlD* gene expression in rice imbibed with *mtlD* constructs. Top; Primers *mtlD*-U1/*mtlD*-D1 were used for the PCR. The PCR products are shown on a 1.5% ethidium bromide-stained agarose gel after electrophoresis. Bottom; an autoradiogram of the gel hybridized with the *mtlD* gene specific probe. p, positive control (*pGEM-mtlD* DNA as template); n, negative control (*pGEM-4z* as template).

presence of the exogenous *mtlD* gene were observed to express the *mtlD* gene (Fig. 4B). As shown in Fig. 3, one was the rice line #2. These results suggest that the bacterial *mtlD* gene was successfully introduced and transiently expressed in the rice, while, until now, no stable transformant has been made using this experiment. However, several studies have shown that DNA introduced by an imbibition technique can exist for days in plant cells [8, 9, 13, 14]. The presence of the *mtlD* gene in stable transgenic rice has to be confirmed to determine its function as a salt-tolerant gene. When other harsh environment-induced genes were isolated from microorganisms [15], the imbibition technique can be used to examine whether these putative genes can help plants to survive against such stress.

In conclusion, the growth and germination of rice plants transiently transformed with the bacterial gene *mtlD* were

enhanced and these enhancements appeared to be due to the expression of the *mtlD* gene. Accordingly, this imbibition method can be used for testing the function of such putative genes *in vivo* prior to the production of stable transgenic plants.

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