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Ectopic expression of *myo*-inositol 3-phosphate synthase induces a wide range of metabolic changes and confers salt tolerance in rice

Hiroki Kusuda[a], Wataru Koga[a], Miyako Kusano[b,c], Akira Oikawa[b,d], Kazuki Saito[b,e], Masami Yokota Hirai[b], Kaoru T. Yoshida[a,*]

[a] *Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[b] *RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa, Japan*
[c] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan*
[d] *Faculty of Agriculture, Yamagata University, Tsuruoka-shi, Yamagata, Japan*
[e] *Graduate School of Pharmaceutical Sciences, Chiba University, Chuo-ku, Chiba, Japan*

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ABSTRACT

Salt stress is an important factor that limits crop production worldwide. The salt tolerance of plants is a complex biological process mediated by changes in gene expression and metabolite composition. The enzyme *myo*-inositol 3-phosphate synthase (MIPS; EC 5.5.1.4) catalyzes the first step of *myo*-inositol biosynthesis, and overexpression of the *MIPS* gene enhances salt stress tolerance in several plant species. In this study, we performed metabolite profiling of both *MIPS*-overexpressing and wild-type rice. The enhanced salt stress tolerance of *MIPS*-overexpressing plants was clear based on growth and the metabolites under salt stress. We found that constitutive overexpression of the rice *MIPS* gene resulted in a wide range of metabolic changes. This study demonstrates for the first time that overexpression of the *MIPS* gene increases various metabolites responsible for protecting plants from abiotic stress. Activation of both basal metabolism, such as glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle, and inositol metabolism is induced in *MIPS*-overexpressing plants. We discuss the relationship between the metabolic changes and the improved salt tolerance observed in transgenic rice.

1. Introduction

Salt stress caused by high levels of salt in soil is one of the major abiotic stress conditions that limit crop production. Approximately 20% of irrigated land is affected by salt, and the area of the salt-affected land is increasing due to continuing irrigation [1]. Salt stress causes inhibition of the growth of young leaves, stomatal closure, and senescence of mature leaves. Plants have developed various mechanisms to defend against salt stress, including ion homeostasis, osmolyte biosynthesis, compartmentalization of toxic ions, and systems that scavenge reactive oxygen species (ROS) [2].

myo-Inositol is an essential nutrient for plant and animal growth, and is a central compound in diverse biochemical processes, including signal transduction, stress responses, osmoprotection, cell wall biogenesis, growth regulation, indole acetic acid (IAA) metabolism, membrane trafficking, and phytic acid biosynthesis [3,4]. Inositol is crucial for normal plant growth and development. *myo*-Inositol 3-phosphate synthase (MIPS; EC 5.5.1.4) catalyzes the reaction from glucose-6-phosphate (G6P) to *myo*-inositol 3-phosphate, the first step of inositol metabolism. The induction of *MIPS* gene expression by salt stress has been shown in several plant species [5,6]. In addition, overexpression of a *MIPS* gene in transgenic plants led to increased inositol levels and enhanced salt stress tolerance in rice, Arabidopsis, and tobacco [6–8]. MIPS from the halophytic wild rice, *Porteresia coarctata* (Roxb.) Tateoka, (PcINO1) maintains its enzymatic function even in the presence of 500 mM NaCl, and overexpression of *PcINO1* resulted in salt-tolerant transgenic plants [7,9]. A biosynthetic pathway in *P. coarctata* generates the methylated inositol derivative, pinitol, which is a strong osmolyte [10]. This pathway is not

Abbreviations: ABA, abcisic acid; CE–MS, capillary electrophoresis–mass spectrometry; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GABA, gamma aminobutyric acid; GC–MS, gas chromatography–mass spectrometry; MIPS, *myo*-inositol 3-phosphate synthase; NT, non-transformants; PPP, pentose phosphate pathway; RINO1ox, *RINO1*-overexpressor; ROS, reactive oxygen species; TCA, tricarboxylic acid.

* Corresponding author at: Laboratory of Conservation Ecology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan. Tel.: +81 3 5841 8086; fax: +81 3 5841 1306.
E-mail address: ayosida@mail.ecc.u-tokyo.ac.jp (K.T. Yoshida).

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found in rice, Arabidopsis, or tobacco. Therefore, the metabolites and metabolic changes that contributed to salt tolerance in these transgenic plants are obscure because of the existence of divergent pathways beginning from *myo*-inositol 3-phosphate.

Severe suppression of *MIPS* gene expression during seed development resulted in an aberrant embryo structure and impaired germination in transgenic rice [11,12]. This indicates that *myo*-inositol 3-phosphate and/or its derivatives play a crucial role in seed development and that regulation of *MIPS* gene expression may affect various aspects of biological performance.

In this study, we generated transgenic rice plants that overexpressed the rice *MIPS* gene, *RINO1*. We investigated salt tolerance in *RINO1*-overexpressor (RINO1ox) transgenic plants in detail and performed metabolome analysis to identify the mechanism that confers salt tolerance to RINO1ox plants. We discuss the implications of these results in elucidating salt tolerance in RINO1ox plants.

2. Materials and methods

2.1. Plasmid construction

A *RINO1* gene was isolated by differential screening to isolate embryogenesis-abundant genes [13]. Based on sequence and expression analyses of the *RINO1* gene, RINO1 was shown to function as a *myo*-inositol 3-phosphate synthase (MIPS) [4]. The hygromycin-resistant binary vector pBIAct/nos [14] containing the rice *Actin1* promoter [15] and the *nos* terminator was used in this study for construction of *Act::RINO1*. The Gateway reading frame cassette (Invitrogen, Carlsbad, CA) was inserted into the pBIAct/nos *Sma*I site between the *Actin1* promoter and the *nos* terminator. The PCR-amplified *RINO1* coding region was cloned using the Gateway system (Invitrogen). The resulting plasmid was transferred into the *Agrobacterium tumefaciens* strain EHA105 by electroporation. Transgenic rice plants (*Oryza sativa* cv. Kitaake) were produced as described by Kuwano et al. [12,16]. The presence of the introduced genes was confirmed by PCR using specific primer sets for the *Actin1* promoter and the *RINO1* gene. The overexpression of *RINO1* was confirmed by RT-PCR and real-time RT-PCR as described previously [17].

2.2. Growth conditions

The transgenic and wild-type plants were grown on Murashige and Skoog (MS) medium [18] containing 0.1% Gelrite in a pot. Eight-day-old plants in pots were transferred to 0 or 250 mM NaCl/MS solution. After NaCl treatment for a period of 3.5 days, the seedlings were transferred to MS medium solution without NaCl and were grown further. The plants were grown in a growth chamber at 28 °C with 14-h/day illumination of approximately 240 μmol m^{-1} s^{-1}.

2.3. Chlorophyll content

The absolute amounts of chlorophyll a and chlorophyll b were determined by extraction with a large excess of 80% acetone for 30 min with agitation. Centrifugation at 20,000 × *g* for 10 min yielded an acetone supernatant containing chlorophyll. The chlorophyll concentration was determined spectrophotometrically at 645 and 663 nm [19].

2.4. Metabolome analysis

For metabolome analyses, fourth leaf tissue was harvested at 0, 6, and 12 h after NaCl stress induction. As a control, 8-day-old plants were transferred to MS solution without NaCl and fourth leaves were harvested at 0, 6, 12 h after transfer. The harvested leaves were frozen immediately in liquid nitrogen and stored at −80 °C for metabolite profiling. Non-targeted gas chromatography–mass spectrometry (GC–MS) and capillary electrophoresis–mass spectrometry (CE–MS) analyses were performed as described previously [20,21]. Analysis was performed in sextuplicate on six separate tissue preparations from independent samples.

2.5. Ascorbate measurement

All leaf tissue from individual plants was harvested for determination of ascorbate amounts. Ascorbate was extracted from pooled leaves using 6% (w/v) metaphosphoric acid and quantitated as described by Rao and Ormrod [22].

3. Results

3.1. Growth of RINO1ox plants under salt stress

Twenty-seven primary transformants (T_0) were obtained after regeneration from hygromycin-resistant callus. All of the plants set viable T_1 seeds. Because MIPS catalyzes the first step of phytic acid biosynthesis, overexpression of *RINO1* was expected to increase phytic acid content and decrease inorganic phosphate levels in seeds. We selected three transgenic lines whose seeds contained lower inorganic phosphate levels than non-transformants (NT) and confirmed the overexpression of the *RINO1* gene in these transgenic lines. Fixed progeny lines were used in the subsequent experiments.

Overexpression of a *MIPS* gene from the halophyte smooth grass (*Spartina alterniflora*) in transgenic Arabidopsis plants conferred improved salt tolerance [8]. These transgenic plants retained higher chlorophyll contents and exhibited better growth performance under salt stress than NT plants. Therefore, we investigated the chlorophyll contents in RINO1ox and NT plants after 2 days of 250 mM NaCl stress treatment (Fig. 1A). All three independent RINO1ox lines exhibited significantly higher chlorophyll content than the NT plants. The 3–12 progeny line was used in subsequent experiments because of its high expression of *RINO1* (Fig. S1) and its low content of inorganic phosphate in seeds (Fig. S2).

We compared the salt stress tolerance of the NT and RINO1ox lines after 3.5 days of 250-mM NaCl treatment and after 10.5 days of continued growth on medium lacking NaCl. Approximately 75% of the NT plants died, but most of the RINO1ox plants survived (Fig. 1B). In addition, the fourth leaf emerged from the sheath of the third leaf after 3.5 days of 250 mM NaCl treatment in most of the RINO1ox plants, but the fourth leaf did not emerge in the majority of the NT plants (Fig. 2B), even though the germination and seedling growth rates of the RINO1ox and NT plants were identical before the NaCl treatment (Fig. 2A). When 8-day-old seedlings were transferred to medium without NaCl, there was no difference in growth between NT and RINO1ox plants after 3.5 days. Growth of newly expanding leaves was strongly suppressed in the NT plants grown on the medium containing salt. These phenotypes indicated that the RINO1ox plants exhibited higher levels of salt tolerance than the NT plants.

3.2. Metabolome analysis of inositol and related compounds

To identify the metabolic differences between NT and RINO1ox plants underlying the differences in chlorophyll content and growth of the fourth leaves, we performed metabolome analysis on the fourth leaves from plants subjected to NaCl stress. We collected the fourth leaves 0, 6, and 12 h after NaCl treatment. The sizes of the fourth leaves were not significantly different between the NT and RINO1ox plants until 12 h of NaCl treatment (Fig. S3).