Journal of Plant Physiology 168 (2011) 2268–2271

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Short communication

Expression analysis of the gene family associated with raffinose accumulation in rice seedlings under cold stress

Masakazu Saito[1], Midori Yoshida*

NARO Hokkaido Agricultural Research Center, Hitsujigaoka, Sapporo 062-8555, Japan

ARTICLE INFO

Article history:
Received 26 April 2011
Received in revised form 15 July 2011
Accepted 16 July 2011

Keywords:
Cold stress
Galactinol
Raffinose
Rice
Seed imbibition protein

ABSTRACT

A considerable increase in the concentration of raffinose was detected in rice seedlings exposed to chilling for more than 4 days. The content of raffinose in leaf blades increased from hardly detectable levels (0–1 days) to approximately 9 mg/g FW after 11 days of chilling treatment. Accumulation occurred in leaf blades but not in sheaths. Analysis of the expression of candidate genes related to galactinol and raffinose synthesis revealed that transcript levels of two galactinol synthase and four raffinose synthase genes increased in leaf blades before the accumulation of raffinose became detectable.

Introduction

Raffinose is synthesized by raffinose synthase (RS; EC 2.4.1.82), which transfers a galactose residue from galactinol to sucrose and is one of the osmoprotectants involved in several environmental stresses, particularly drought stress. Galactinol is synthesized from UDP-galactose and *myo*-inositol by galactinol synthase (GolS; EC 2.4.1.123). *Arabidopsis* overexpressing the *GolS* gene had increased contents of both galactinol and raffinose and showed tolerance to drought stress (Taji et al., 2002). Enzymatic activity and mRNA level of *GolS* in *Arabidopsis* are increased by cold (Liu et al., 1998). There have been many studies showing that the expression of genes related to biosynthesis of galactinol and raffinose is involved in several stresses, and expression specificity of the *GolS* genes to different stresses has also been reported in *Arabidopsis*. In rice, accumulation of raffinose in seedlings overexpressing *OsWRKY11*, which is related to heat shock and drought stress, was reported (Wu et al., 2009). It has also been reported that raffinose content in cold-tolerant rice was slightly increased by chilling treatment at 13/10 °C for 4 days (Morsy et al., 2007) and that the expression of a *GolS* homologous gene was induced in rice seedlings exposed to a treatment of 4 °C for 2–15 h (Phan et al., 2010). However, the genes related to the induction of raffinose synthesis by chilling stress in rice, which has many homologous genes of *GolS* and *RS* have not been identified, and the involvement of *OsRS* genes in cold stress has not been shown. The objective of this study was to identify the genes that play a role in accumulation of raffinose in rice plants exposed to cold stress.

Materials and methods

Plant material and growth conditions

Rice seeds (*Oryza sativa* L. cv. Yukihikari) were immersed in distilled water for 3 days at 28 °C for germination, sown in soil in a plastic container, and then transferred to a growth chamber under the conditions of 25 °C/20 °C and a 15-h light/9-h dark cycle (photoenergy: 150 μmol m^{-2} s^{-1} photon flux density). Fourteen-day-old seedlings were exposed to a temperature of 5 °C with continuous light (10 μmol m^{-2} s^{-1} photon flux density). For analyses, leaf blades and leaf sheaths were sampled at 0, 1, 4, 7 and 11 days of chilling treatment.

Quantitative real-time PCR analysis

Total RNA was extracted from each sample using an RNeasy plant mini kit (Qiagen USA, Valencia, CA). After treatment with DNase I (Invitrogen, amplification grade) to remove a trace amount of DNA, cDNA was synthesized from 1 μg of total RNA using SuperScript®III (Invitrogen) with an oligo-dT_{20} primer. The

Abbreviations: AGA, alkaline α-galactosidase; GolS, galactinol synthase; ORF, open reading frame; RFO, raffinose oligosaccharide; RS, raffinose synthase; SIP, seed imbibition protein.

* Corresponding author. Tel.: +81 11 857 9524; fax: +81 11 859 2178.
E-mail address: midori@affrc.go.jp (M. Yoshida).
[1] Present address: Innovation Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8503, Japan.

0176-1617/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2011.07.002

transcript levels of candidate genes were quantified by real-time RT-PCR using the 7300 Real Time PCR System (Applied Biosystems) and SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio) according to the instructions of manufacturers. Gene-specific primers were designed around the end of the ORF sequences including the 3′-UTR sequence so that the resulting PCR products had approximately equal sizes of 100 bp. Primer pairs used in the real-time PCR are shown in Supplementary Table 1. The specificity of products was validated by dissociation curve analysis. The quantification analysis was performed by the standard curve methods using serial dilutions of plasmid-cloned specific amplicons. The thermal cycle used was as follows: 95 °C for 10 s, 50 cycles at 95 °C for 15 s and 60 °C for 35 s. Amplification of specific sequences of both the targeted gene and internal control gene for all sampling days was performed in the same 96-well plate. Results were obtained from three individual plants and analyzed using SDS software ver. 1.4 (Applied Biosystems). The expression levels of target genes were normalized with the value of a polyubiquitin gene (Moritoh et al., 2005) as a constitutively expressed reference gene.

Carbohydrate extraction and analysis

Total water-soluble carbohydrates were extracted as described by Yoshida et al. (1998) in boiling deionized water containing 0.9 mg/mL stachyose as an internal standard. Total carbohydrates were analyzed by high-performance anion exchange chromatography (HPAEC) on a DX 500 chromatograph (Dionex, Sunnyvale, CA, USA) with a CarboPac PA-1 anion exchange column and a pulsed amperometric detector (PAD). Peaks for *myo*-inositol, galactinol, glucose, fructose, sucrose and raffinose were identified and calculated by comparison with authentic standards (Wako Pure Chemicals, Osaka, Japan).

Results

Carbohydrate contents and water contents in rice exposed to cold stress

Changes in galactinol and raffinose contents showed similar patterns. Galactinol and raffinose were mainly accumulated in leaves, and contents of both galactinol and raffinose suddenly increased after 4 days under the cold condition. In leaf sheath tissues, galactinol and raffinose were hardly detected or little accumulated until 7 days of chilling treatment (Fig. 1). The amount of raffinose increased greatly and the content exceeded the levels of glucose and fructose after 11 days of chilling treatment. The content of *myo*-inositol, which is both a substrate in galactinol synthesis and a product in raffinose synthesis, was higher in leaf blades than in leaf sheaths under control growth conditions, and the content in leaf sheaths slightly increased with chilling treatment (Fig. 1). Raffinose is thought to accumulate under the condition of drought stress. Since cold stress might cause osmotic stress as a result of cellular water deficit, the water contents in leaf blades and sheaths of rice were measured. During chilling treatment, the water contents in leaf blades and sheaths were both stable at around averages of 78.3% and 86.3%, respectively.

Analysis of the expression of GolS and RS genes in rice under the cold condition

Candidates of raffinose synthesis-related genes, *GolS* and *RS*, were identified using the *O. sativa* genome database (RAP-DB) and database of the National Center for Biotechnology Information by a homologue search against *wsi76* (D26537, Takahashi et al., 1994; Os07g0687900), which is annotated as *GolS*, and against *O. sativa RS* gene (BAD68247; Li et al., 2007), respectively. The

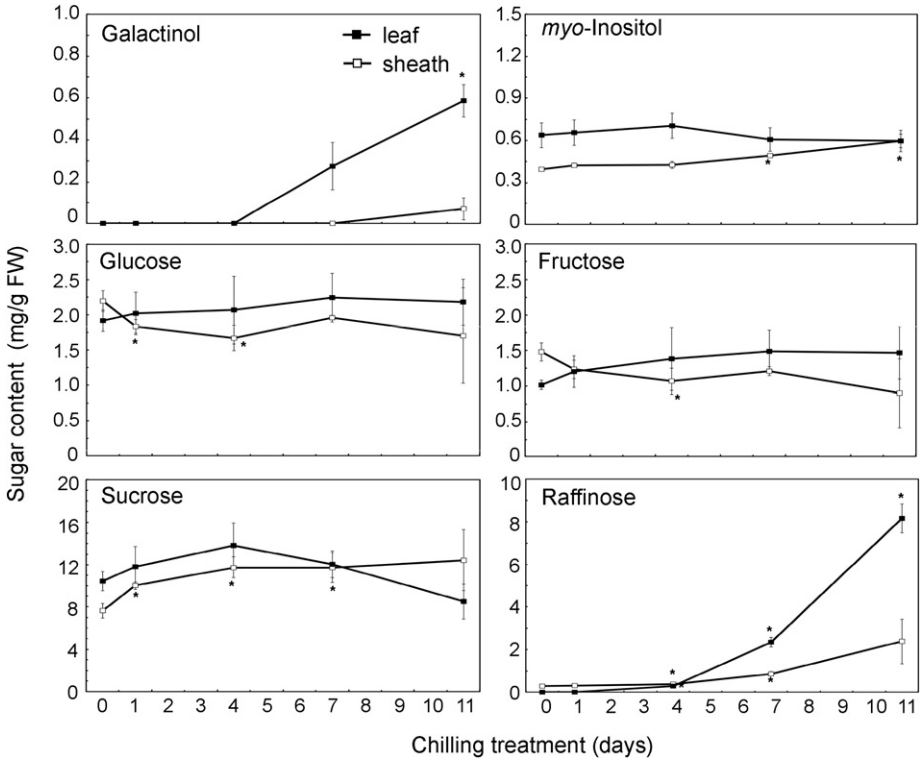

Fig. 1. Changes in the concentrations of soluble carbohydrates in leaf blades and sheaths of 14-day-old rice seedlings during cold stress. Fourteen-day-old seedlings were exposed to a chilling (5 °C) temperature. Total sugars were prepared from leaf blades (closed square) and sheaths (open square), and the levels of individual sugars were determined by HPAEC-PAD. Data shown are means ± SD ($n = 3$). Significant differences in the data for chilling-treated seedlings on each day compared to non-treated seedlings at day 0 were analyzed by Student's *t*-test. Asterisks, * indicate the value at $P < 0.05$.