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Galactinol is Involved in Induced Systemic Resistance against Bacterial Infection and Environmental Stresses

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Abstract - We previously demonstrated that root colonization of the rhizobacterium, *Pseudomonas chlororaphis* O6, induced expression of a galactinol synthase gene (*CsGolS1*), and resulting galactinol conferred induced systemic resistance (ISR) against fungal and bacterial pathogens in cucumber leaves. To examine the role of galactinol on ISR, drought or high salt stress, we obtained T-DNA insertion *Arabidopsis* mutants at the *AtGolS1* gene, an ortholog of the *CsGolS1* gene. The T-DNA insertion mutant compromised resistance induced by the O6 colonization against *Erwinia carotovora*. Pharmaceutical application of 0.5 - 5 mM galactinol on roots was sufficient to elicit ISR in wild-type *Arabidopsis* against infection with *E. carotovora.* The involvement of jasmonic acid (JA) signaling on the ISR was validated to detect increased expression of the indicator gene *PDF1.2.* The T-DNA insertion mutant also compromised tolerance by increasing galactinol content in the O6-colonized plant against drought or high salt stresses. Taken together, our results indicate that primed expression of the galactinol synthase gene *AtGolS1*in the O6-colonized plants can play a critical role in the ISR against infection with *E. carotovora,* and in the tolerance to drought or high salt stresses.

Key words - drought, *Erwinia carotovora*, galactinol, high salt, jasmonate, induced systemic resistance, rhizobacteria

Introduction

Induced systemic resistance (ISR) elicited by root-colonizing bacteria (rhizobacteria) capable of reducing disease in aboveground plant parts through a plant-mediated mechanism has been demonstrated in many plant species. A trait of ISR is effective against a broad spectrum of plant pathogens, including oomycetes, fungi, bacteria and viruses (Van Loon *et al*., 1998). These large numbers of rhizobacteria thrive on the surface or inside of the plant root where nutrients (plant exudates) are provided. A common feature of ISR caused by these microorganisms is priming enhanced defense. In primed plants, defense responses are accelerated upon pathogen attack instead of being directly activated, resulting in enhanced resistance to the pathogen (Conrath *et al.*, 2002). ISR is often associated with enhanced expression of jasmonic acid (JA)/ethylene (ET)-responsive genes in *Arabidopsis* (Van Wees *et al*., 2008). Certain beneficial rhizobacteria primes affected plants by enhancing salicylic acid (SA)-dependent defenses. However, ISR-expressing plants are primed for enhanced expression of predominantly JA- and ET-regulated genes upon pathogen infection (Van Wees *et al*., 2008). Although JA/ET has emerged as an important regulator of rhizobacteria-mediated ISR, one must consider other signaling components that enhance defense cooperatively with JA/ET in order to elucidate the signal transduction pathways involved in ISR.

The rhizobacterium *Pseudomonas chlororaphis* O6 elicited ISR in tobacco and cucumber plants, specifically by demonstrating protective effects against two foliar bacterial pathogens, *P. syringae* pv. tabaci and *Erwinia carotovora* subsp. *carotovora* (Spencer *et al*., 2003). *P. chlororaphis* O6 also provides protection against the leaf spot fungus *Corynespora cassiicola* (Kim *et al.,* 2004). Suppressive subtractive hybridization was applied in order to gain insight into the O6-mediated

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ISR against this pathogen, resulting in isolation of the cucumber galactinol synthase (*CsGolS1*) gene (Kim *et al*., 2008). The expression level of *CsGolS1* and the resulting galactinol content were increased in plants challenged by *C. cassiicola* and subjected to several hours of O6 treatment. However, no difference was detected in unchallenged water control. *CsGolS1*-overexpressing transgenic tobacco plants demonstrated constitutive resistance against the pathogen *Botrytis cinerea* as well as increased levels of galactinol. The pharmaceutical application of galactinol in wild-type tobacco plants enhanced resistance against pathogen infection while stimulating the accumulation of defense-related gene transcripts (Kim *et al*., 2008). These results may suggest that galactinol acts as an endogenous molecular signal for the induction of O6-mediated ISR against fungal pathogens in plants. Among known 10 independent *Arabidopsis* galactinol synthase genes, only *AtGolS1* was specifically induced upon infection with the fungal pathogen *B. cinerea* and the *AtGolS1* gene was primed by O6 colonization against the pathogen in *Arabidopsis* leaves (unpublished data). By the interaction between *Arabidopsis* and *B. cinerea,* it was demonstrated that expression of the *AtGolS1* gene by O6 colonization is mediated through the jasmonate-dependent pathway, stimulating ISR in *Arabidopsis* against the fungal pathogen (unpublished data). Recently, Taji *et al*. (2002) demonstrated that overexpression of *AtGolS2* resulted in enhanced accumulation of endogenous galactinol and also showed improved drought tolerance in plants, suggesting that galactinol may function in drought-stress tolerance. An analysis of the expression of the *CsGolS1* gene under various abiotic stress conditions revealed that expression of the *CsGolS1* gene is not increased in cold- and dark-treated cucumber leaves, but high salinity and dehydration stress treatments strongly induced transcription of the gene in the same leaves (Kim *et al*., 2008). Increased accumulation of *CsGolS1* transcript in the *CsGolS1*-transgenic tobacco also induced a high level of tolerance against drought and high salinity (250 mM NaCl) conditions.

Here, we demonstrate that, using the T-DNA inserted knockout mutant, primed expression of the galactinol synthase gene *AtGolS1*in the *P. chlororaphis* O6-colonized plants can play a critical role in the ISR against infection with *E. carotovora,* and in the tolerance to drought or high salt stresses.

Materials and Methods

Plant materials

The parental Arabidopsis ecotype Columbia (Col-0) and the *Arabidopsis AtGolS1* T-DNA insertion lines, SALK-121059 and SALK-128044, were obtained from the *Arabidopsis* T-DNA insertion collection of the Salk Institute (Alonso et al. 2003). In order to select plants homozygous for the T-DNA insertion, the gene-specific primers (forward and reverse) 5'-AAACCGCTGATGCTATGTCC-3' and 5'-AACCAAC-GACTCCTTTCACG-3', as well as 5'-GGAAAGAAGCGA-ACATGGAG-3' and 5'-GGTGATGAACGGCTTCAGAT-3' were used for the SALK-121059 and SALK-128044 insertion lines, respectively. Plant seeds were surface-sterilized by soaking in 70% ethanol for 2 min and followed by soaking in 1% sodium hypochlorite for 20 min. Seeds were rinsed four times in sterile distilled water and maintained for 3 days in darkness at 4℃ to maintain germination uniformity. Sterile seeds were individually added to the surface of the medium (2:1:1 mixture of peat moss, vermiculite, and perlite) in pots measuring 10.5 × 10.5 × 9 cm 15-30 seeds per pot. Every 2 days, the plants were watered with 20 ml of sterile water per pot. Seedlings were grown under a 16h light/8h dark cycle under 80μmol photons $m^{-2}s^{-1}$. The temperature was maintained at 22 ± 1℃ with a relative humidity of 50-60%.

Rhizobacterial inoculation and bacterial pathogen infection

To prepare the rhizobacterial inoculum, the *P. chlororaphis* O6 strain was grown overnight in King's medium B (KB) broth. Cells were then pelleted by 10 min of centrifugation at 10,000×g, washed once in sterile water, and resuspended to 1×10^8 cfu/ml. Two-week-old Arabidopsis plants grown in soiled pots were treated with 35 ml of O6 strain suspension.

Three-week-old *Arabidopsis* plants cultivated in soiled pot were inoculated with *Erwinia carotovora* SCC1 by spraying bacterial suspension (1×10^8 cfu/ml) onto the leaves. Three days after inoculation, soft rot disease was rated by counting the number of symptomatic plants per pot. The bacterium was streaked onto King's medium B agar plate that contained 2% agar and incubated at 28℃ for 2 days. To prepare inoculums, the bacteria were grown overnight in KB broth, pelleted by centrifugation at 10,000×g for 10 min, washed once with

sterile water, and resuspended to 1×10^8 cfu/ml. Three independent experiments were performed, with at least 100 plants per experiment.

Sugar treatment

Galactinol dehydrate was purchased from Fluka Co, raffinose from Wako Pure Chemical Industries, Ltd, and stachyose tetrahydrate (98%) from Aldrich Co. Sterile sugar solutions at various concentrations, were used to treat *Arabidopsis* plants growing in sterilized medium. *Arabidopsis* seeds were placed on 0.5 % (w/v) MS agar supplemented with 1.5 % sucrose in each well of a 12-well microtiter plate (SPL Korea). After three weeks, 10 μl of sugar solution was applied to the growing plant roots present on MS medium in each well. Three days after inoculation, soft rot disease was rated by counting the number of symptomatic plants per plate.

Drought and high salinity stress treatments

Three-week-old Arabidopsis plants grown in soiled pots were soaked in 125 mM NaCl solution for high salinity treatment and then cultivated in a growth chamber. Control plants of the same age were soaked in distilled water. For drought treatment, water was withheld from the three-week-old plants grown in soiled pots until the plants showed visible wilting. Three independent experiments were performed, with at least 50 plants per treatment, respectively.

Sugar analysis by HPLC

Arabidopsis leaves were frozen with liquid nitrogen, crushed, added to 20 ml of 50% methanol, and shaken for 1h. The apparatus utilized for HPLC analysis was an LC 2000 series (Jasco, Japan) coupled with an evaporative light scattering detector, ELSD 200 (Softa, USA). An ELSD nitrogen generator was employed as a source of nitrogen gas. Separation was conducted at 30℃ on a reversed-phase Shim-pack CLC-NH_2 column (6 mm×150 mm). ELSD conditions were optimized for maximum sensitivity: the spray chamber temperature was set at 35℃ and the drift tube temperature was set to 60℃. The mobile phase was 70% acetonitrile, which was maintained at a constant flow-rate of 1 ml/min, and the injection volume was 40 μl. The carbohydrates were determined quantitatively via the comparison of peak areas of the chromatograms with those of the standards.

Real-time RT-PCR

PCR primer sets approximately 120bp in length were designed using the GeneRunner program for use in real-time RT-PCR (Table 1), as was suggested by the manufacturer (Qiagen). In order to detect expression of the *AtGolS1::T-DNA* gene in T-DNA insertion *Arabidopsis* mutants, specific primer sets for SALK121059 (Forward: 5'- GAAGCGAACATGGAGAGGG -3'; Reverse: 5'- AGCGGACGGTGCG-

Table 1. Gene-specific primers used for real-time RT-PCR analysis

Gene	AGI No.	Primer sequences (5' to 3')
AtGolS1	At2g47180	TCCACCGTGACGATAACAAA and TTGGCTAAACCAACGACTCC
PR1	At2g14610	GGCCTTACGGGGAAAACTTA and ACTTTGGCACATCCGAGTCT
VSP	At5g24780	GGAGATGCGGTGAAGATATATGC and GTTGGTGTGACAAAATGGAAGC
HEL	At3g04720	CGGCAAGTGTTTAAGGGTGAAG and TGCTACATCCAAATCCAAGCCT
PDF1.2	At5g44420	AATGGATCCATGGCTAAGTTTGCTTCCATC and AATGAATTCAATACACACGATTTAGCACC
β-Actin	At2g37620	CATCAGGAAGGACTTGTACGG and GATGGACCTGACTCGTCATAC

GTCACG -3') and for SALK128044 (Forward: 5'- TCCAC-CGTGACGATAACAAA -3' Reverse: 5'- GATCCACAA-GTATACGACGG -3') were used. For the detection of RNA transcripts in various samples, real-time quantification of RNA targets was conducted in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, Sydney, Australia) using a QuantiTect SYBR Green RT-PCR kit (Qiagen). In accordance with the manufacturer's instructions (Qiagen), the reaction mixture (20 μl) contained 200 ng of total RNA, 0.5 μM of each primer, and appropriate amounts of enzymes and fluorescent dyes. For the control reaction, no RNA was added, resulting in the lack of detectable fluorescence. Real-time PCR results were captured and analyzed using Rotor-Gene 3000 version 6.0.16 software (Corbett Research). Transcript levels were normalized to the expression of *β-ACTIN* measured in the same samples.

Statistics

Data were analyzed by ANOVA using JMP 4.0 software (SAS Institute; Cary, NC). The significance of observed values was determined by Duncan's multiple range test (P =0.05). Three independent experiments were performed, with at least 50 plants per experiment.

Results and Discussion

Functional role of the *AtGolS1* gene for resistance against *E. carotovora*

We have previously demonstrated that colonization of *Pseudomonas chlororaphis* O6 on plant roots primes the expression of the cucumber galactinol synthase (*CsGolS1*) gene, and that the resulting galactinol induces systemic resistance against the fungal pathogen, *Corynespora cassiicola* (Kim *et al*. 2008). Transgenic tobacco plants overexpressing CsGolS1 demonstrated constitutive resistance against the pathogens *Botrytis cinerea* and *Erwinia carotovora,* in addition to increased accumulation in galactinol content (Kim et al. 2008). Among 10 independent *Arabidopsis* galactinol synthase (*AtGolS*) genes, only *AtGolS1* was specifically induced upon infection with the fungal pathogen *B. cinerea* (Unpublished data). Furthermore, only *AtGolS1* was primed against pathogen by O6 colonization in the leaves of *Arabidopsis,* suggesting that the *Arabidopsis AtGolS1* gene is an ortholog of the cucumber *CsGolS1* gene (Unpublished data). Relative expression of the *AtGolS1* gene was also clearly induced upon infection with *E. carotovora* to a level higher in this experiment (Fig. 1A). Specifically, expression of the *AtGolS1* gene was increased approximately 13-fold 16h after inoculation with the fungal pathogen (Fig.1A). Expression of the

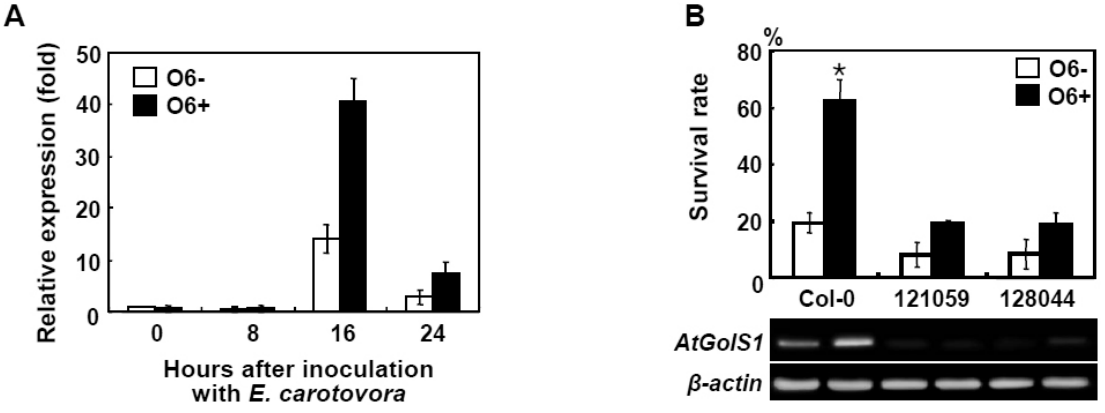

Fig. 1. Differential expression of the *AtGolS1* gene in *Arabidopsis e*cotype Col-0 with (O6+) and without (O6-) *P. chlororaphis* O6 colonization on roots 0, 8, 16 and 24h after challenge inoculation with *E. carotovora.* The relative mRNA levels of the *AtGolS1* genes normalized with respect to housekeeping gene *β-actin*. B: Reduction in survival rate in the T-DNA insertion Arabidopsis mutants at the *AtGolS1* gene (121059 and 128084) against *E. carotovora* infection. Three-week-old plants grown in soiled pots with (O6+) and without (O6-) strain O6 colonization on roots, were used to estimate the survival rate against the bacterial infection. Vertical bars indicate ± standard deviation. Bars with asterisk denote a significant difference at P=0,05 between O6- and O6+.

AtGolS1 gene was also primed upon O6 treatment and challenge with *E. carotovora*, as evidenced by significantly higher transcription compared to water control plants. Accumulation of *AtGolS1* transcripts in the leaves of non- colonized water control (O6) *Arabidopsis* plants was the highest 16h following inoculation with *E. carotovora* (Fig. 1A). These results indicate that expression of the *AtGolS1* gene is primed to respond more strongly when exposed to bacterial pathogen.

Arabidopsis mutant lines in the *AtGolS1* gene (SALK121059 and SALK128044) were obtained, and homozygous lines were generated. Southern blotting analysis was conducted to confirm the insertion of T-DNA in the genome of each mutant line (data not shown). In order to confirm whether *AtGolS1* was disrupted by mutation, we measured expression of *AtGolS1* in two independent *Arabidopsis AtGolS1::T-DNA* mutant lines by quantitative real-time RT-PCR using primer sets (Table 1) specific for the *AtGolS1::T-DNA* genes. RT-PCR analysis showed that the transcript levels of *AtGolS1* in the two mutant lines were decreased compared with that of wild-type Col-0 (Fig. 1B). O6 colonization in the roots increased the level of *AtGolS1* gene expression to levels significantly higher than those in wild type, but was not detectable in the two mutant lines (Fig. 1B). The two mutant lines showed no alterations in plant growth, development, or fertility, but did seem to have reduced survival rates upon infection with *E. carotovora.* Enhanced survival rates as the result of O6 colonization (O6+) were also dramatically decreased in mutant lines compared with that observed in wild-type Col-0 (Fig. 1B). Therefore, mutants were highly susceptible to *E. carotovora* infection, suggesting that root colonization by *P. chlororaphis* O6 plays a critical role in the *AtGolS1* gene regulation during ISR.

Pharmaceutical application of galactinol protects *Arabidopsis* from bacterial infection

If disease resistance is enhanced in plants when the endogenous levels of galactinol are increased, one would expect that exogenous galactinol treatment should be active in protecting *Arabidopsis* against *E. carotovora* infection. To test this hypothesis, purchased galactinol and other raffinose family oligosaccharides (RFOs) such as raffinose and stachyose, were tested for its ability to enhance resistance to the bacterial infection on wild-type *Arabidopsis*. As controls, plants were treated with distilled water or sucrose (as a negative control), respectively. Feeding galactinol to wild-type Arabidopsis through the root system in MS agar resulted in a significant disease resistance on the upper portions of the plant. In order to find the optimum concentration of exogenously treated galactinol, various levels of galactinol (from 0.1 mM to 10 mM) were applied onto the plant roots of wild-type Arabidopsis seedlings growing in MS agar. The most effective concentration in terms of survival rate against *E. carotovora* infection was seen at 0.5 mM (Fig. 2). When 0.5 mM galactinol was applied to Arabidopsis roots and the upper portions of the plants were subsequently challenged with *E. carotovora*, survival rate by the bacterium was increased clearly when compared with water-treated plants (Fig. 2). However, there was a limitation in differentiating the induced resistance between galactinol and the other disaccharides by measuring the disease lesion areas. When 5 mM raffinose was applied to Arabidopsis roots and then upper portions of the plants were subsequently challenged with *E. carotovora,* the trisaccharide also clearly conferred enhanced disease resistance against the bacterial infection (Fig 2). On the other hand, sucrose and stachyose did not confer resistance in the plants. The increase of galactinol content in plant leaves, both endogenously through the transgene expression and exogenously through the sugar treatment, conferred disease resistance against bacterial infection. These findings support the theory that galactinol may function as a signaling molecule for induction of disease resistance.

Jasmonic acid-dependent regulation of the ISR

E. carotovora infection in *Arabidopsis* plants both with (O6+) and without (O6) strain O6 colonization on roots 0, 8, 16 and 24h after challenge inoculation, induces expression of *Arabidopsis* defense response genes such as *HEL*, *PDF1.2, VSP1* and *PR1. PR-1* is generally used as a marker for the activation of the SA signaling pathway, whereas *PDF1.2* and VSP are used as markers for JA signaling (Berger *et al*., 1995; Takahashi *et al.*, 2004). The gene transcripts of *HEL, PDF1.2* and *VSP* were found to be increased, but *PR1* do not, 16h after *E. carotovora* infection, indicating the involvement of JA or ET signaling during ISR. However, ET-inducible *HEL* gene

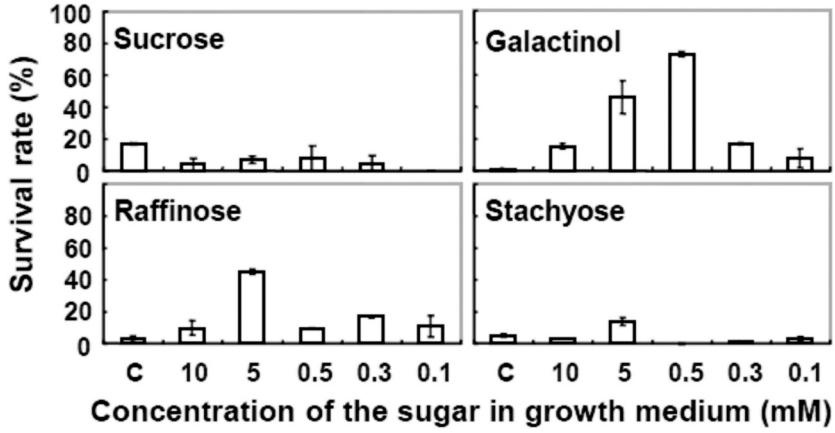

Fig. 2. Disease resistance induced in wild-type *Arabidopsis* by exogenous treatment of galactinol and raffinose on roots in MS medium against infection with *E. carotovora.* The control plant (indicated by the letter C) was treated with distilled water and challenged with the pathogen.

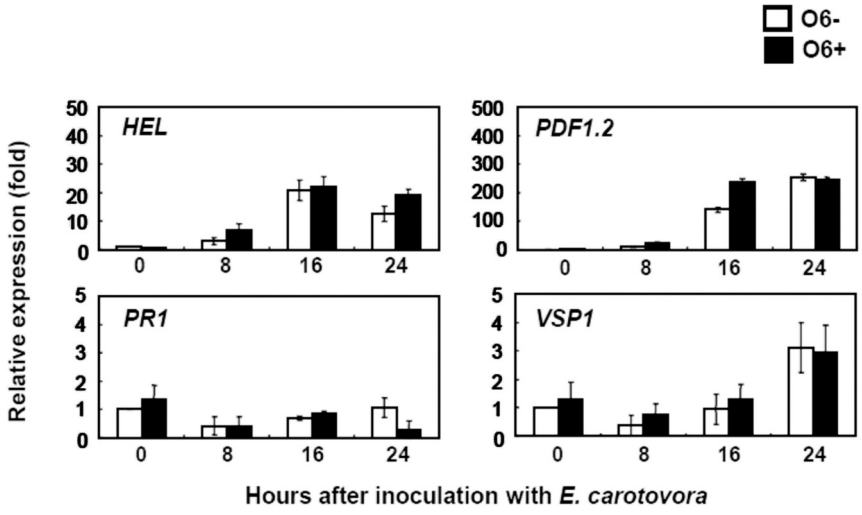

Fig. 3. Differential expression of the *HEL, PDF1.2, PR1* and *VSP1* genes in *Arabidopsis* plants with (O6+) and without (O6-) strain O6 colonization on roots 0, 8, 16 and 24h after challenge inoculation with *E. carotovora.* The specific expression pattern of each gene was determined by real-time RT-PCR analysis using the gene specific primer set. Vertical bars indicate ± standard deviation.

expression (Potter *et al.*, 1993) and JA-inducible *VSP1* was not primed in O6 colonized wild-type Col-0 (Fig. 3). Only PDF1.2 gene expression was primed by O6 root colonization (Fig. 3). These results indicate that signal transduction in ISR may be a jasmonate-dependent pathway. It is known that JA plays an important role in induced resistance, referred to as rhizobacteria-mediated ISR, triggered by beneficial microorganisms such as non-pathogenic fluorescent *Pseudomonas* spp. (Pieterse *et al.*, 2000). Galactinol-mediated signaling during ISR in *Arabidopsis* was dependent on the JA signaling pathway but was independent of the SA pathway.

Galactinol enhanced tolerance to drought and high salinity stresses

RFOs consist of galactose units linked to sucrose (Peterbauer *et al.*, 2001). Galactinol synthase catalyzes the synthesis of galactinol from UDP-D-galactose and myo-inositol (Sprenger and Keller, 2000). Beside its known function as a galactosyl donor that synthesizes RFO and operates as an osmoprotectant in response to drought stress (Taji *et al.*, 2002), Taji *et al.* (2002) demonstrated that overexpression of *AtGolS2* resulted in enhanced accumulation of endogenous level of galactinol and raffinose, and improved drought tole-

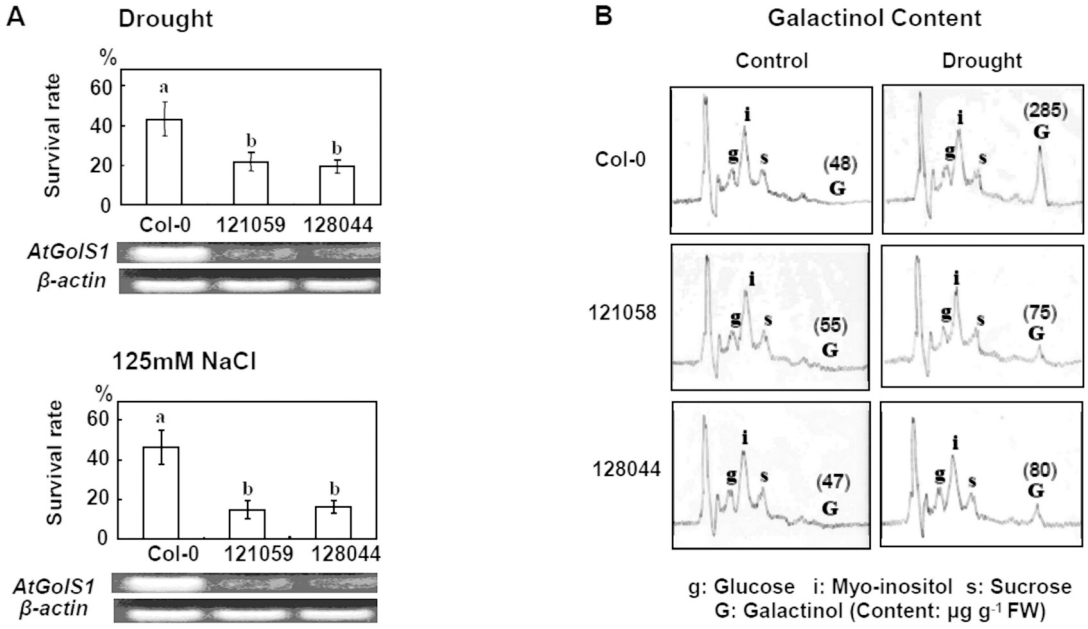

Fig. 4. A: Decrease of survival rate in the *AtGolS1::TDNA* mutants after drought and 125 mM NaCl treatment. Three-week-old mutant grown in soiled pots were used to estimate the survival rate after the treatments. Vertical bars indicate ± standard deviation. Bars with different letters denote a significant difference at $P < 0.05$. B: HPLC profiles of some carbohydrate metabolites purified in the Col-0 and *Arabidopsis AtGolS1::TDNA* mutant after drought treatment. The number in parenthesis above the G letter is galactinol content (μg g-1 Fresh Weight) quantified from the HPLC profile.

rance in transgenic *Arabidopsis*. These results indicate that galactinol has a function in drought-stress tolerance in plants. Expression analysis of the *CsGolS1* gene under various abiotic stress conditions revealed that drought and high salinity conditions strongly induced transcription of this gene in cucumber plants (Kim *et al.*, 2008). In accordance with the result of Taji et al. (2002), the increased level of galactinol in the *CsGolS1*-transgenic tobacco conferred a high level of tolerance to drought and high salinity (250 mM NaCl). The T-DNA insertion mutant also compromised tolerance induced by increased galactinol content in the O6-colonized plant against drought or high salt stresses (Fig. 4). In order to determine whether decreased level of survival rate to drought stress is coupled to the decreased *de novo* production of galactinol within Arabidopsis leaves, we assessed the accumulation of endogenous galactinol levels by high performance liquid chromatography (HPLC) in the T-DNA mutant leaves. No difference in galactinol level was detected between control and T-DNA mutant plants prior to drought treatment. As is shown in Fig. 4B, however, several days after drought treatment, the galactinol content in the control leaves was found to be approximately 4-fold higher than that detected in T-DNA mutant leaves.

Acknowledgments

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