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Galactinol Is a Signaling Component of the Induced Systemic Resistance Caused by *Pseudomonas chlororaphis* O6 Root Colonization

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Root colonization by *Pseudomonas chlororaphis* O6 in cucumber elicited an induced systemic resistance (ISR) against *Corynespora cassiicola*. In order to gain insight into O6-mediated ISR, a suppressive subtractive hybridization technique was applied and resulted in the isolation of a cucumber galactinol synthase (*CsGolS1*) gene. The transcriptional level of *CsGolS1* and the resultant galactinol content showed an increase several hours earlier under O6 treatment than in the water control plants following *C. cassiicola* challenge, whereas no difference was detected in the plants without a pathogen challenge. The *CsGolS1*-overexpressing transgenic tobacco plants demonstrated constitutive resistance against the pathogens *Botrytis cinerea* and *Erwinia carotovora*, and they also showed an increased accumulation in galactinol content. Pharmaceutical application of galactinol enhanced the resistance against pathogen infection and stimulated the accumulation of defense-related gene transcripts such as *PR1a*, *PR1b*, and *NtACS1* in wild-type tobacco plants. Both the *CsGolS1*-overexpressing transgenic plants and the galactinol-treated wild-type tobacco plants also demonstrated an increased tolerance to drought and high salinity stresses.

Plants have evolved a variety of mechanisms by which to defend themselves against pathogens. In addition to their constitutive barriers, plants can induce resistance against pathogenic attack upon appropriate stimulation prior to contact with the pathogens. Thus far, two types of induced resistance have been defined on the basis of differences in their stimulation: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Ryals et al. 1996; Van Loon et al. 1998). SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA), coupled with the concomitant activation of a set of SAR genes (Ryals et al. 1996; Sticher et al. 1997). ISR, elicited by rhizobacteria, is capable of reducing disease in aboveground plant parts, and this has been demonstrated in a variety of plant species (Kloepper et al. 2004; Van Loon et al. 1998). The rhizobacterium *Pseudomonas chlororaphis* O6 elicited ISR in tobacco and cucumber plants, demonstrating protective effects against two foliar bacterial pathogens, *P. syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora* (Radtke et al. 1994; Spencer et al. 2003), and providing protection against the leaf spot fungus *Corynespora cassiicola* (Kim et al. 2004), respectively.

The signal transduction pathway of rhizobacteria-mediated ISR is known to be distinct from that of SAR. A rhizobacterium, *P. fluorescens* WCS417r, has been demonstrated to induce ISR via an SA-independent signaling pathway (Pieterse et al. 1996, 2002; Ton et al. 2002; Van Wees et al. 1997). Furthermore, components of the jasmonic acid (JA) and ethylene (ET) responses were required for the triggering of WCS417r-mediated ISR (Knoester et al. 1999; Pieterse et al. 1998). Several other rhizobacterial strains, including *P. fluorescens* CHA0 (Iavicoli et al. 2003), *P. fluorescens* 89B61 (Yan et al. 2002), *P. putida* WCS358r (Van Wees et al. 1997), *Serratia marcescens* 90–166 (Press et al. 1997; Ryu et al. 2003), and *Bacillus pumilus* SE34 (Ryu et al. 2004; Yan et al. 2002) have been shown to induce SA-independent but ET- or JA-dependent signaling pathways for protection against plant pathogens. The exposure of the tobacco seedlings to *P. chlororaphis* O6 has been shown to stimulate the accumulation of transcripts for plant defense genes, including *PR-1g*, 3-hydroxy-3-methyglutaryl coenzyme A reductase (*HMGR*), and lipoxygenase (*LOX*), but did not stimulate the accumulation of transcripts of the *PR-1a* gene (Spencer et al. 2003). This result suggests that strain O6 can activate the JA or ET plant defense pathways

Verhagen and associates (2004) investigated the transcriptional response of over 8,000 *Arabidopsis* genes during rhizobacteria-mediated ISR in an effort to identify ISR-related genes; however, none of the tested genes evidenced a consistent change in leaf expression in response to the effective colonization of the roots by WCS417r. After a challenge inoculation with the bacterial speck pathogen *P. syringae* pv. *tomato* on the WCS417r-induced plants, 81 genes evidenced augmented expression patterns within the leaves (Verhagen et al. 2004). This result indicates that these genes were primed to respond faster or more strongly when exposed to pathogenic attack. The capacity for augmented defense expression is referred to as "priming" and this phenomenon has been demonstrated in different plant species against different pathogens, insects, and even abiotic stresses (Conrath et al. 2002). *P. chlororaphis* O6-mediated ISR was also associated with the fast induction of several genes after a challenge inoculation with *C. cassiicola* in cucumber leaves relative to water-treated controls (Kim et

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al. 2004). However, the physiological and molecular mechanisms underlying this priming phenomenon remain to be thoroughly elucidated. This lack of data compelled us to determine the genes involved in the priming phenomenon in plants.

The raffinose family oligosaccharides (RFO), including raffinose and stachyose, are common in the plant kingdom. RFO are not accumulated in vegetative tissues other than seeds, and their synthesis is induced in vegetative tissues during dehydration stress (Blackman et al. 1992; Brenac et al. 1997; Downie et al. 2003; Horbowicz and Obendorf 1994; Taji et al. 2002). Functions of RFO, other than their involvement in the desiccation tolerance of seeds, have been suggested to include transporting carbohydrates within the phloem (Ayre et al. 2003), the protection of plant cells against heat stress (Penikulangara et al. 2004), and protection from freezing (Pennycooke et al. 2003; Sprenger and Keller 2000). However, the specific function or functions of RFO in portions of plants other than the seeds remain largely unknown. The biosynthesis of RFO is preceded by the reversible addition of galactose units from galactinol to sucrose, and chain elongation is catalyzed via the consecutive action of raffinose synthase and stachyose synthase (Bachmann et al. 1994). The galactose donor, galactinol, is produced from UDP-Gal and *myo*-inositol via galactinol synthase (GolS) during the initial step in the biosynthesis of RFO. Galactinol has not been demonstrated to have any other function in plants other than as a galactosyl donor for RFO synthesis (Sprenger and Keller 2000). Three stress-responsive *GolS* genes (*AtGolS1, 2*, and *3*) were recently identified in *Arabidopsis*. *AtGolS1* and *AtGolS2* were shown to be induced by drought and high-salinity stresses but not by cold stress. In contrast, *AtGolS3* was induced by cold stress but not by drought or salt stress (Taji et al. 2002). Transgenic plants that overexpressed *AtGolS2* cDNA demonstrated an enhanced level of galactinol and raffinose within leaves and evidenced improved drought tolerance (Taji et al. 2002). *AtGolS1* gene expression was both drought stress inducible and heat inducible, and it was shown that the raffinose content within leaves increased upon heat stress in the wild-type strains but not in the *AtGolS1* mutants (Penikulangara et al. 2004).

In this study, we have demonstrated that *P. chlororaphis* O6-mediated ISR is associated with the primed expression of a galactinol synthase gene in plants. The gene was primed to respond faster and more strongly when exposed to pathogenic attack. The endogenous increase in galactinol contents within the plant leaves induced by O6 colonization or exogenously by pharmaceutical application conferred disease resistance against pathogen attack. To the best of our knowledge, this study is the first report to demonstrate that a simple disaccharide, galactinol, can function as a signaling factor for ISR against plant pathogens.

RESULTS

Expression of a galactinol synthase (*CsGolS1*) gene is primed in the leaves of cucumber plants by *P. chlororaphis* O6 root colonization.

Root colonization by *P. chlororaphis* O6 in cucumber elicited ISR against the fungal leaf spot disease caused by *C. cassiicola* (Kim et al. 2004) and the bacterial angular leaf spot disease caused by *P. syringae* pv. *lachrymans*, respectively (data not shown). An effort to detect specific genes involved in the *P. chlororaphis* O6-mediated ISR resulted in the isolation of several genes that demonstrated no consistent changes in expression levels in response to effective root colonization by the O6 strain. However, following a challenge inoculation with *C. cassiicola* onto the ISR-expressing cucumber leaves, these genes evidenced augmented expression patterns, indicating a much faster response time after pathogenic attack. For example, the transcript of the *Cucumis sativus* ISR gene 3 (*CsISR3*) clone, encoding a cucumber galactinol synthase (CsGolS1), was accumulated after a challenge inoculation with *Corynespora cassiicola*. The increased accumulation of the gene transcript occurred several hours earlier in the O6-colonized, challenge-inoculated (O6+) leaves compared with only the challenge-inoculated (O6–) leaves (Fig. 1A). The *CsGolS1* gene transcript accumulated in O6+ plants as early as 0 to 12 h after inoculation, whereas this has been shown to occur after 12 to 24 h in O6– plants (Fig. 1A). These expression levels were also verified by real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis (data not shown). On the cucumber leaves that were challenge inoculated with the bacterial pathogen *P. syringae* pv. *lachrymans*, the accumulation of the *CsGolS1* gene transcript also increased far more rapidly in time and achieved a higher level in the O6+ plants than in the O6– plants (Fig. 1A). The initiation of *CsGolS1* gene transcription was increased from 12 h after bacterial challenge in the O6+ leaves, whereas no increases was detected in the O6– leaves until 24 h after the challenge inoculation.

In order to determine whether enhanced levels of *CsGolS1* gene expression are coupled to the increased de novo production of galactinol within cucumber leaves, we assessed the accumulation of endogenous galactinol levels by high-performance liquid chromatography (HPLC) in cucumber leaves in O6– and O6+ plants. Three different time points (0, 12, and 24 h) following the challenge inoculation with *C. cassiicola* were recorded. No differences were detected between treated and untreated plants in terms of the galactinol content on cucumber leaves prior to challenge with *C. cassiicola*. However, 12 h after challenge inoculation, the galactinol content in the O6+ cucumber leaves was found to be approximately 3.3-fold higher than that detected in O6– leaves (Fig. 1B). Interestingly, the increased levels of galactinol content due to O6-mediated ISR faded within 12 h and, by 24 h following the challenge inoculation, the galactinol content had essentially declined to basal levels (Fig. 1B). The enhanced transcriptional level of the *CsGolS1* gene in the O6+ cucumber leaves at 12 h persisted until 36 h after pathogen challenge (Fig. 1A); therefore, further investigations should be conducted regarding the sudden decrease in galactinol content observed at 24 h. On the other hand, expression of the *CsGolS1* gene at 24 h in the O6– cucumber challenged with the fungal pathogen does not coincide with elevated galactinol levels (Fig. 1B). Even when we divided the time points more narrowly (0, 8, 16, and 24 h after inoculation), elevated galactinol level was not detected in either the O6– or O6+ and challenged cucumber (data not shown). These facts suggest that an increased level of galactinol by the fungal infection may be used up very quickly to be a substrate for the synthesis of RFO and, possibly, to be a signal molecule for the defense signaling pathways (for example; inositol 1,4,5-triphosphate cycle and so on). If this is true, it is probable that the increased galactinol level can be detected only for a short period of time during the initial stage of ISR. Quantitative determination demonstrates that the amount of the *CsGolS1* transcript at 24 h in O6– plants is far more than that at 12 h in O6+ plants (data not shown); therefore, increased galactinol level in O6– plants may be detectable for a certain period of time between 16 and 24 h.

Overexpression of the *CsGolS1* gene confers constitutive resistance in transgenic tobacco plants against fungal and bacterial pathogens.

To support the functional role of galactinol in ISR, we generated transgenic tobacco plants in which the sense *CsGolS1* transcript was constitutively overexpressed. We selected four

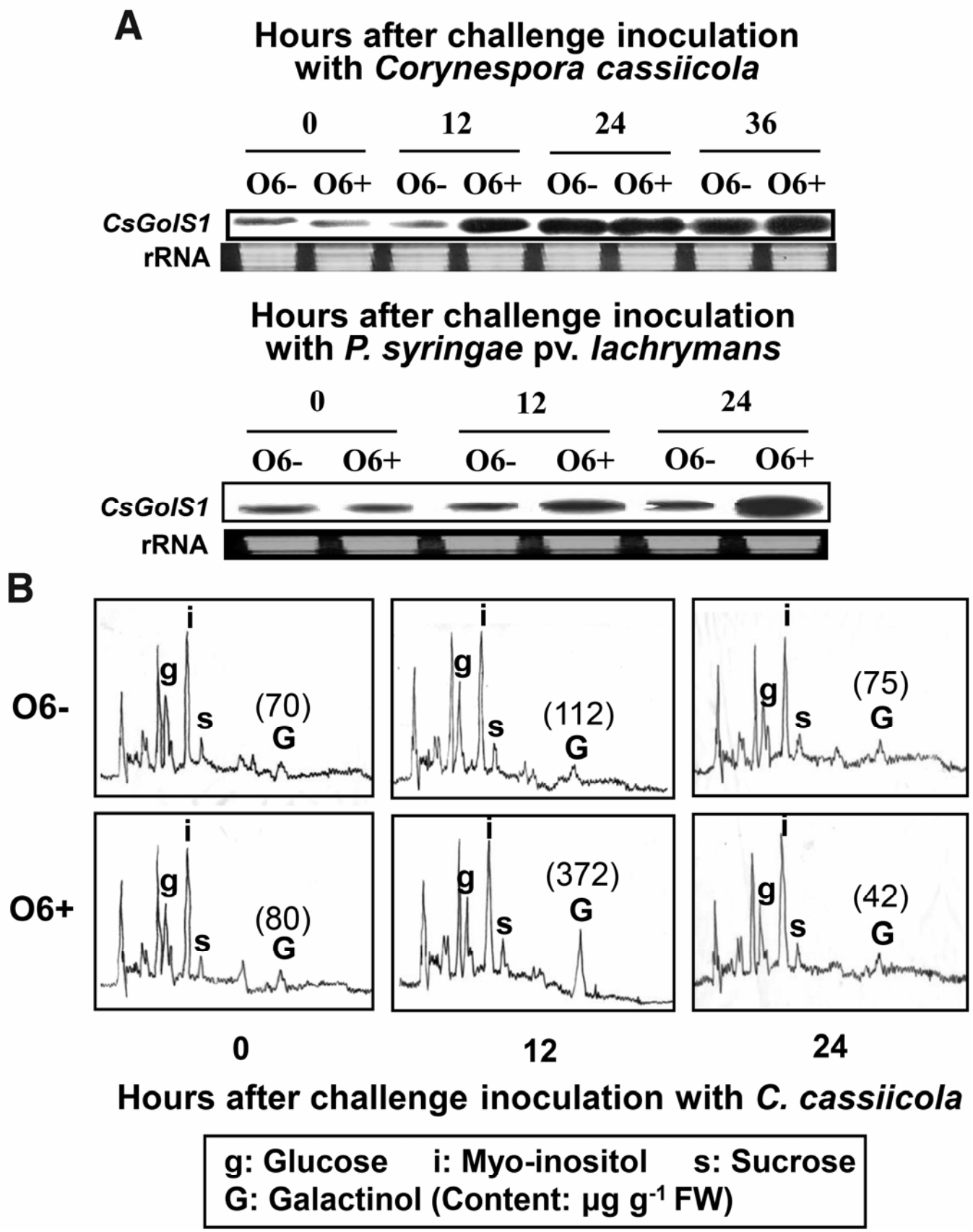

Fig. 1. A, Differential expression of the *CsGolS1* gene in cucumber leaves with (O6+) and without (O6–) *Pseudomonas chlororaphis* O6 colonization on the roots 0, 12, 24, and 36 h after challenge inoculation with *Corynespora cassiicola* or with *P. syringae* pv. *lachrymans*. Expression pattern of the *CsGolS1* gene was determined by Northern hybridization using a gene-specific DNA probe. **B,** High-performance liquid chromatography (HPLC) profiles of some carbohydrate metabolites purified from cucumber leaves with (O6+) and without (O6–) strain O6 colonization on roots 0, 12, and 24 h after challenge inoculation with *C. cassiicola*. The number in parentheses above the G letter is the galactinol content (µg g^{-1} fresh weight) quantified from the HPLC profile.

independent transgenic T_1 lines that showed an increased expression of the *CsGolS1* gene compared with wild-type and vector-only plants. The four T_1 lines were morphologically indistinguishable from the wild-type plants and showed endogenous levels of galactinol approximately 6- to 10-fold higher than that of wild-type plants (Fig. 2A). In order to evaluate the range of protection offered by overexpression of the *CsGolS1* gene, we examined selected T_1 lines for disease resistance to fungal pathogen *Botrytis cinerea* and to bacterial pathogen *E. carotovora* subsp. *carotovora* of tobacco. After pathogen inoculation, survival rates were assessed by determining the number of symptomatic plants per pot. Increased accumulation of galactinol content by the stable integration of the *CsGolS1* gene into the tobacco genomes conferred disease resistance constitutively against both *B. cinerea* and *E. carotovora*. At 3 to 5 days after inoculation with *B. cinerea*, the *CsGolS1* overexpressors clearly showed more resistance to the pathogen infection (40 to 63% increase in the survival rate) compared with wild-type plants, although some variation was shown among the T_1 lines (Fig. 2B). We also examined the responses of the transgenic tobacco against infection with *E. carotovora* to estimate the functional role of this gene in the bacterial disease resistance. Infection of wild-type tobacco with the bacterial pathogen destroyed approximately 93% plants (7% survival rate), whereas 51 to 72% of the transgenic tobacco plants survived the bacterial infection (Fig. 2B).

Pharmaceutical application of galactinol protects tobacco plants from pathogen infection and induces the expression of defense-related genes.

If disease resistance is enhanced in the transgenic tobacco plants when the endogenous levels of galactinol are increased, one would expect that exogenous galactinol treatment should be active in protecting wild-type tobacco plants against pathogen infection. To test this hypothesis, purchased galactinol was tested for its ability to enhance resistance to bacterial and fungal infections on wild-type tobacco plants. As controls, plants were treated with distilled water or with other disaccharides (negative control) such as sucrose and lactose. Feeding galactinol to wild-type tobacco plants through the root system in Murashige-Skoog (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 to 10 mM) were applied onto the plant roots of wild-type tobacco 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. 3A). Galactinol feeding of wild-type tobacco through the root system in MS agar resulted in fungal resistance on the upper portions of the plant. When 0.5 mM galactinol was applied to tobacco roots and the upper portions of the plants were subsequently challenged with *B. cinerea*, the disease lesion area caused by the fungus was reduced significantly when compared with water-treated plants (Fig. 3B). However, there was a limitation in differentiating the induced resistance between galactinol and the other disaccharides by measuring the disease lesion areas. On the other hand, when 0.5 mM galactinol was applied to tobacco roots and then upper portions of the plants were subsequently challenged with *E. carotovora*, the disaccharide galactinol clearly conferred enhanced disease resistance against the pathogen infection compared with distilled water or other disaccharide controls. (Fig. 3C). These outcomes in conjunction with the above results allow us to prove that galactinol treatment induces disease resistance in plants against fungal and bacterial pathogens, and the galactinol-mediated induction is not due to the osmotic effect that can be possibly caused by such sugar treatment.

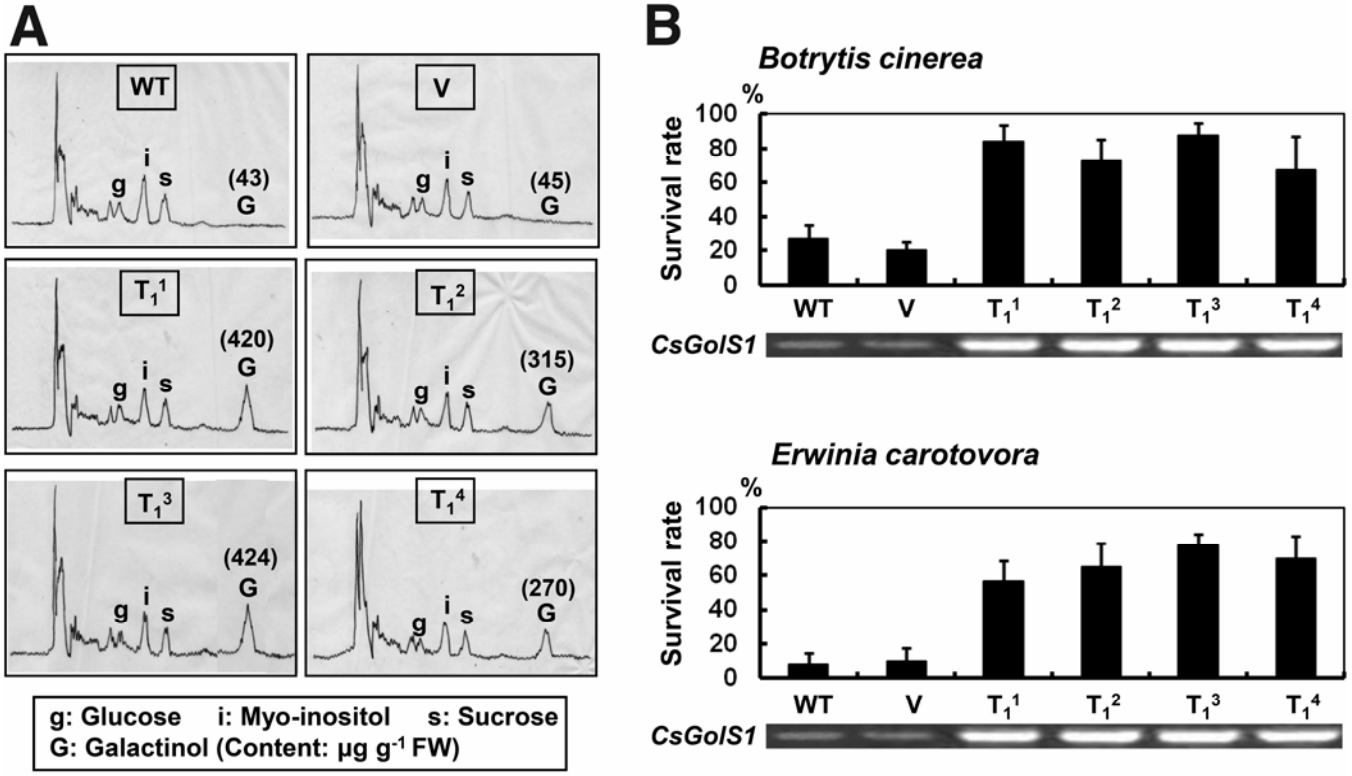

Fig. 2. A, High-performance liquid chromatography (HPLC) profiles of some carbohydrate metabolites purified from the *CsGolS1*-overexpressing transgenic tobacco tissue. The number in parentheses above the G letter is the galactinol content (μg g^{-1} fresh weight) quantified from the HPLC profile. WT: wild type, V: vector only, T_1^1, T_1^2, T_1^3, and T_1^4: four independent T_1 lines. **B,** Increase in survival rate of the *CsGolS1*-overexpressing tobacco T_1 lines against pathogen infections. Five-week-old transgenic plants grown on soil in pots were infected with *Botrytis cinerea* or *Erwinia carotovora*. Vertical bars indicate ± standard deviation.

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 and fungal infections. These findings support the theory that galactinol may function as a signaling molecule for induction of disease resistance. In order to test this hypothesis, we decided to investigate the galactinol responsiveness of those defense-related genes such as *PR1a*, *PR1b*, *Nicotiana tabacum* 1-aminocyclopropane-1-carboxylic acid synthase 1 (*NtACS1*), and *N. tabacum* 1-aminocyclopropane-1-carboxylic acid synthase 2 (*NtACS2*). Feeding of 0.5 mM galactinol through the root system resulted in an increased accumulation of defense-related gene transcripts in the leaves of wild-type tobacco (Supplemental Fig. 1). Because the level of transcript accumulation by 0.5 mM galactinol treatment was relatively low, the expression of the defense-related genes altered by 5 mM galactinol treatment was investigated. The pattern of increased transcript accumulation after treatment with 5 mM galactinol was very similar to the results obtained by 0.5 mM galactinol. The 5 mM galactinol treatment through the root system induced *PR1a*, *PR1b*, and *NtACS1* gene expression but failed to induce *NtACS2* gene expression in the leaves of wild-type tobacco plants (Fig. 4). This suggested that galactinol might be a signaling component of the defense response to challenging pathogens.

Galactinol-enhanced tolerance to drought and high-salinity stresses.

Taji and associates (2002) demonstrated that overexpression of *AtGolS2* resulted in enhanced accumulation of endogenous level of galactinol and raffinose and improved drought tolerance 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 (data not shown). In accordance with the result of Taji and associates (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) (Fig. 5A). Feeding galactinol to wild-type tobacco plants through the root system in MS agar also resulted in tolerance of the upper portions of the plant to drought and high-salinity conditions (Fig. 5B). Therefore, an increase in the endogenous level of galactinol can protect plants both from biological attacks by pathogens and from damages caused by environmental stresses.

DISCUSSION

Plants resist pathogen attacks by virtue of a broad range of defense mechanisms. Many inducible defense responses are involved in the expression of host resistance, which suggests that they can be elicited by both parasite-specific and nonspecific signals (Hammond-Kosack and Jones 2000; Heath 2000; Mysore and Ryu 2004). Inducible defenses can be activated in plants upon the recognition of pathogen-derived cell wall fragments, including microbial β-(1,3)-glucans and chitin-derived oligomers (Boller 1995; Ebel 1998; Ebel and Mithöfer 1998; Nürnberger et al. 2004). Inducible defense responses are also elicited by host cell wall fragments (including the plant pectin fragments) released during pathogen attack. The fragments perform important functions in plants as signaling molecules involved in the recognition of pathogen attack (Côté and Hahn 1994; Ebel 1998; Ebel and Mithöfer 1998). Many such exogenous cell wall fragments have been shown to be poly- or oligosaccharides. α-(1,4)-Oligogalacturonides (OG) derived from the plant cell wall during pathogen attack are well-known elicitors of defense responses (Côté and Hahn 1994; Ridley et al. 2001). Although the majority of defense responses elicited by OG require a degree of polymerization (DP) between 10 and 15, smaller oligomers with a DP of 2 to 8 have also been shown to activate defense responses (Weber et al. 1996; Wegener et al. 1996), ET production (Simpson et al. 1998), the elicitation of genes involved in JA synthesis (Norman et al. 1999), and the accumulation of protease inhibitors (Moloshok et al. 1992). In our study, an increase in the endogenous galactinol level, a disaccharide responsible for the synthesis of RFO, enhanced the resistance of plants against fungal and bacterial

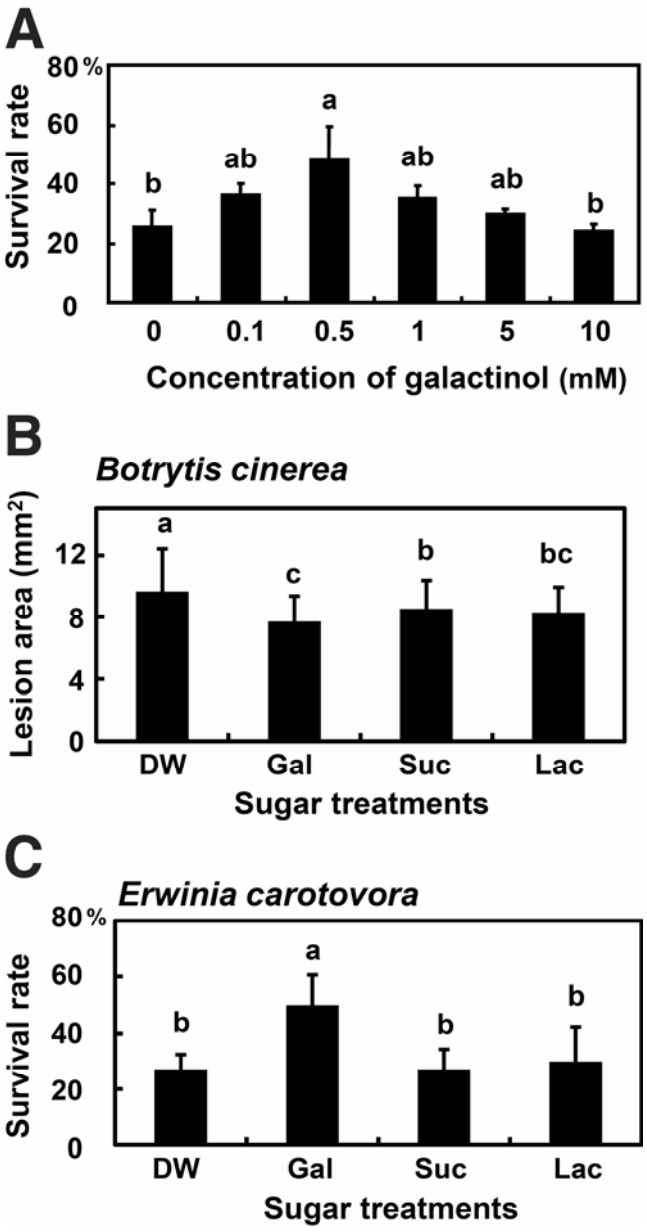

Fig. 3. Induction of resistance in the upper portion of wild-type tobacco against infection with *Botrytis cinerea* and *Erwinia carotovora* by 0.5 mM galactinol feeding through the plant root system in Murashige-Skoog (MS) agar. **A,** Optimum concentration of galactinol in MS agar for the increase in survival rate against *E. carotovora*. **B,** Resistance against *B. cinerea* was quantified by measuring the disease lesion area formed 3 days after inoculation. The inoculation was conducted by pipetting 5 μl of the conidial suspensions (2.5×10^5 spores/ml) onto each leaf. **C,** Resistance against *E. carotovora* was quantified by the survival rate. Vertical bars indicate ± standard deviation. Bars with different letters denote a significant difference ($P \leq 0.05$). DW = distilled water, Gal = galactinol, Suc = sucrose, Lac = lactose.

infections. Our results show that the endogenous synthetic oligosaccharides in the anabolic pathway for RFO (Sprenger and Keller, 2000), including galactinol, can perform important functions as signaling molecules and elicit defense responses against pathogen attack. Pretreatment of rice plants with sucrose enhanced resistance to *Magnaporthe oryzae* infection, and this suggests that sucrose may act as an endogenous molecular signal for induction of defense responses (Gómez-Ariza et al. 2007). However, exogenous sucrose treatment did not protect wild-type tobacco plants against bacterial infections in this experiment (Fig. 3C). Further investigations should be conducted regarding the reason for this result. This may be a simple matter of plant difference (mono- and dicotyledonous), pathogen difference (fungal and bacterial), or the concentration of the applied sugar because galactinol demonstrated an optimum concentration for the induction of resistance.

Since Wei and associates (1991) and Van Peer and associates (1991) reported that certain strains of rhizobacteria activate the plant defense responses against fungal pathogens *Colletotrichum orbiculare* and *Fusarium oxysporum*, a variety of studies have also reported this elicitation of ISR-mediated plant defense. The application of the rhizobacterium *P. chlororaphis* O6 to the roots elicited ISR in the cucumber and tobacco plants against different fungal and bacterial pathogens (Kim et al. 2004; Spencer et al. 2003). ISR-expressing plants have been shown to be primed for the augmented expression of a specific set of pathogen-responsive genes (Conrath et al. 2002; Verhagen et al. 2004). Cucumber plants colonized with *P. chlororaphis* O6 were also primed against *Corynespora cassiicola* infection and demonstrated an augmented expression of identified *CsISR* genes, including a galactinol synthase (*CsGolS1*) gene. The transcription of the *CsGolS1* gene was accelerated by the priming process (Fig. 1A) and the accumulation of the gene transcript coincided with increased galactinol levels in the challenge-inoculated leaves (Fig. 1B). However, the accelerated accumulation of galactinol observed 12 h after the challenge inoculation diminished very rapidly and fell back to basal levels within 12 h. These results imply that the accelerated accumulation of galactinol can function transiently as a signaling molecule during the initial interaction between primed plant leaves and fungal penetration. Galactinol synthase catalyzes the formation of galactinol from UDP-galactose and myo-inositol (Sprenger and Keller 2000). Hence, the enhanced activity of the enzyme in O6-mediated ISR will likely exert an effect on the inositol 1,4,5-trisphosphate (IP3) cycle and the phospholipid composition of the plasma membrane. Phosphoinositide-specific phospholipase C was activated transiently but IP3 contents were shown to have been reduced in soybean cells infected with the bacterial pathogen *P. syringae* pv. *glycinea* (Shigaki and Bhattacharyya 2000). This suggested that reduced IP3 content following infection may result in the suppression of various cellular housekeeping activities, thus diverting the cellular resources either to the synthesis of defense-related compounds or to pathogen growth.

The RFO consist of galactose units linked to sucrose via α-(1,6) glycosidic linkages (Peterbauer et al. 2001). Galactinol synthase (GolS) (EC 2.4.1.123) catalyzes the synthesis of galactinol (*o*-α-D-galactopyranosyl-(1,1)-l-myo-inositol) from UDP-D-galactose and myo-inositol (Sprenger and Keller 2000). Aside from its known functions as a galactosyl donor that synthesizes RFO and operates as an osmoprotectant in response to drought stress (Taji et al. 2002), an enhanced level of galactinol content in plant leaves was demonstrated in this study to signal the induction of disease resistance against pathogens. Galactinol fed hydroponically to tobacco seedlings induced expression of tobacco defense response genes such as *PR1a* (SA-dependent) (Ryals et al. 1996), *PR1b* (JA- or ET-dependent) (Thomma et al.1998), and *NtACS1* (ET-dependent) (Chen et al. 2003) in leaves (Fig. 4). Colonization by the *P. chlororaphis* O6 caused increased accumulation for transcripts of regulated tobacco de-

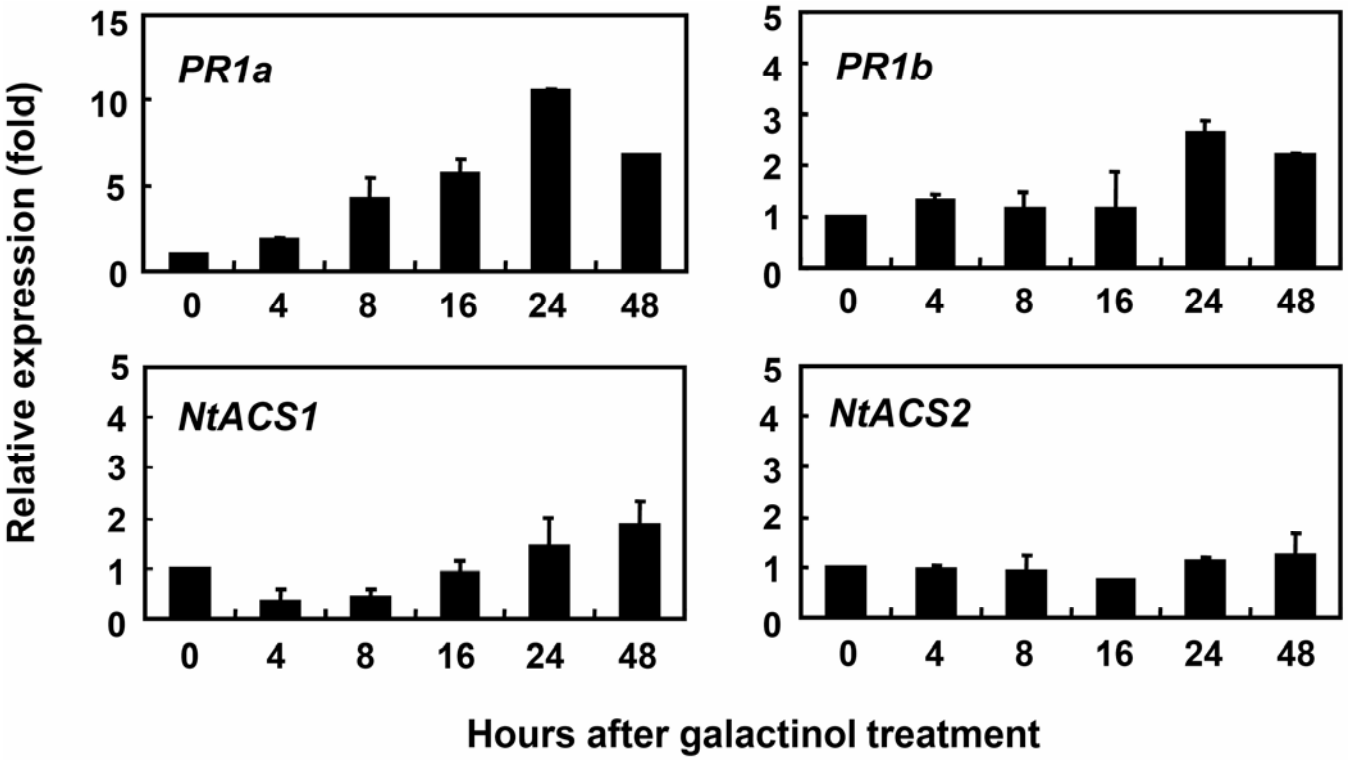

Fig. 4. Expression kinetics of defense-related genes (*PR1a*, *PR1b*, *NtACS1*, and *NtACS2*) in the upper portion of wild-type tobacco, by feeding galactinol of 5 mM through the plant roots. Gene expression was analyzed by real-time reverse-transcription polymerase chain reaction 0, 4, 8, 16, 24, and 48 h after the sugar treatment on the plant root. The specific primer set was used to detect the expression pattern of each gene. Vertical bars indicate ± standard deviation.

fense genes such as *PR-1g, HMGR*, and *LOX* but no accumulation of transcript of the *PR-1a*, a typical marker of SA-mediated responses (Spencer et al. 2003). Therefore, it was suggested that strain O6 may activate the JA or ET plant defense pathways but not an SA-dependent signaling pathway. On the other hand, treatment of tobacco plants with galactinol induced not only *PR1b* and *NtACS2* gene expressions that are for JA and ET pathways but also *PR-1a* gene expression for SA pathway in this experiment (Fig. 4). The reason that the reaction to the *PR-1a* gene is different in the above two experiments can be explained like this. Recently, we demonstrated that root colonization of *Arabidopsis* with the strain O6 induces tolerance to drought stress. Increases in free SA after drought stress of the strain O6-colonized plants suggested a primary role for SA signaling in

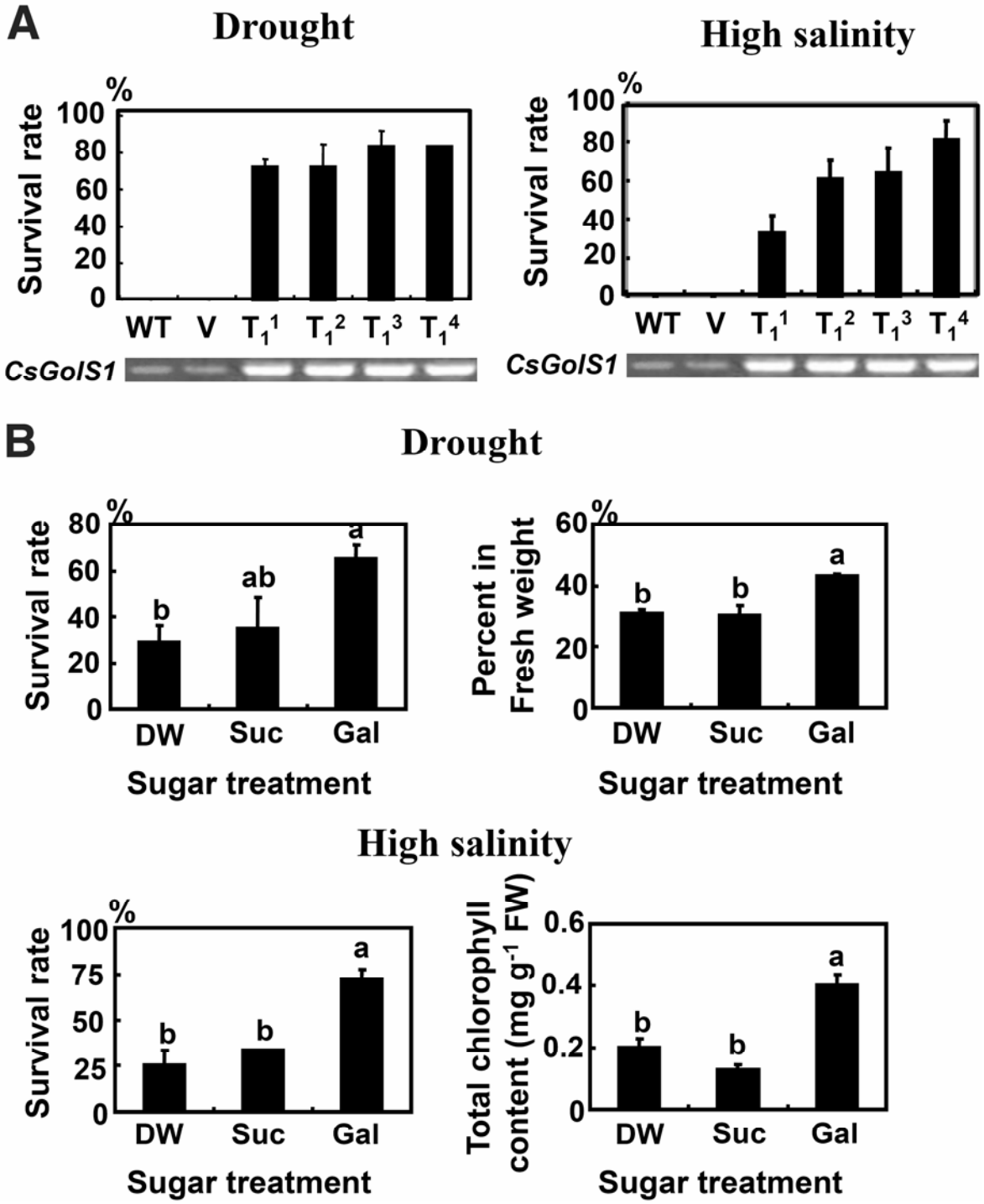

Fig. 5. A, Increase in survival rate of the *CsGolS1*-overexpressing transgenic tobacco (T_1) lines against drought and high-salinity stresses. Five-week-old transgenic plants grown on soil in pots were soaked in 250 mM NaCl solution for high-salinity treatment. For drought treatment, water was withheld from plants until they showed visible wilting. WT: wild type, V: vector only, T_1^{1-4}: four independent T_1 lines. B: Induction of tolerance in the upper portion of wild-type tobacco against drought and high-salinity stresses by 0.5 mM galactinol feeding through the root system in MS agar. The induced tolerance was quantified by the survival rate and percent fresh weight (%) for drought stress, and by the survival rate and total chlorophyll content for high salinity in the same plant. Vertical bars indicate ± standard deviation. Bars with different letters denote a significant difference ($P \leq 0.05$). DW: distilled water, Suc: sucrose, Gal: galactinol.

induced drought tolerance (Cho et al. 2008). Because galactinol treatment also induces drought tolerance (Fig. 5), it is not surprising that the treatment also induced the expression of the *PR-1a* gene in this experiment.

These findings suggest that a high level of endogenous galactinol may be responsible for the earlier and stronger expression of the tobacco defense genes. The fact may retroactively suggest that *P. chlororaphis* O6-mediated priming in cucumber leaves against challenging pathogens is linked to galactinol signaling. Definitive proof that galactinol has a role in disease resistance in plants comes from results obtained in disease resistance studies with galactinol-treated tobacco plants (Fig. 3). This observation confirms the implication that galactinol-dependent defense responses function in the protection of tobacco against *B. cinerea* and *E. carotovora*, and supports the idea that galactinol-mediated priming of defense responses in cucumber plants results in disease resistance against *C. cassiicola* and *P. syringae* pv. *lachrymans*. In this context, our results suggest that galactinol acts as an endogenous molecular signal for induction of defense responses in plants. Kenfield and Strobel (1981) showed that naturally occurring galactinol and raffinose binds to α-galactoside-binding proteins isolated from the cellular membranes of plants. Such binding proteins can be involved in defense responses, and the characterization of the proteins will be an essential first step in clarifying the mechanisms underlying galactinol-mediated ISR.

MATERIALS AND METHODS

Rhizobacterial inoculation on cucumber seed.

The rhizobacterium *P. chlororaphis* O6 was grown on King's medium B (KB) agar plates for 24 h at 28°C. The bacterial cells were subsequently collected and resuspended in 0.02 M phosphate buffer (pH 7.0) and then an equal volume of 2% methylcellulose (Sigma, St. Louis) was added. The bacterial suspension was mixed with cucumber seed (*Cucumis sativus* L., cv. Baekseong), resulting in 5×10^8 to 1×10^{10} CFU/seed, and was air dried overnight in a laminar flow hood (Raupach and Kloepper 1998). Prior to mixing with strain O6, the cucumber seed were surface sterilized with a solution of 10% hydrogen peroxide in order to eliminate seed-infesting organisms. Each cucumber seed coated with the O6 strain was planted to a depth of approximately 1 cm in a 10-cm^2 polyvinyl pot containing 500 cm^3 of sterilized soilless growing medium (peat moss:vermiculite, 7:3). The plants were cultivated in a growth chamber with a light and dark cycle of 10 and 14 h (25 and 20°C), respectively, at 70% relative humidity, with daily watering. One week after sowing, when the cotyledons had sprouted, 20 ml of the rhizobacterial suspension (1×10^8) in sterile water was applied once around the seedling in each pot.

Inoculation of cucumber plants with the pathogen.

Four-week-old cucumber plants in pots were challenge inoculated with *Corynespora cassiicola* by spraying a conidial suspension of the fungus at a concentration of 5×10^4 spores/ml of sterile water, and were maintained for 12 h in a growth chamber at 100% relative humidity. The fungus was grown on Czapek solution agar (Difco, Detroit) plates for 2 weeks at 28°C in an incubator. The aerial mycelia were removed by gently rubbing the surface with a writing brush in tap water and the samples were then air dried in a laminar flow hood. In order to produce the conidia, the plates were positioned in an incubator for 1 week at 28°C with illumination by fluorescent lamps (approximately 2,000 Lux). Four-week-old cucumber plants were challenge inoculated with *P. syringae* pv. *lachrymans* by spraying with a bacterial suspension of 1×10^8 CFU/ml. The bacterial cells were grown at 28°C with shaking at 200 rpm in Luria-Bertani (LB) broth to an optical density at 600 nm of 2.0, centrifuged, and resuspended in sterile water.

Tobacco transformation.

The sense *CsGolS1* DNA of cucumber was synthesized by PCR using two synthetic primers, left 5′ GCTCTAGAAAGTT AATATGTCTCC 3′ (*Xba*I restriction site underlined) and right 5′ GGGGTACCATATACTTAAGCAGCAGA 3′ (*Kpn*I restriction site underlined), and using the *CsGolS1* cDNA as a template. The *CsGolS1* DNA was cloned into the predigested binary vector, pBI 121, replacing the β-glucuronidase gene. Tobacco (*N. tabacum* cv. Xanthi) was transformed by the leaf-disk method using *Agrobacterium tumefaciens* LBA4404 containing the *CsGolS1* recombinant plasmid. Individual kanamycin-resistant regenerated shoots were selected, and the plants were rooted in MS medium with no growth regulators and then transferred to soil. T_1 seed resulting from self-pollination were aseptically germinated in continuous light (2,000 lux) at 26°C on MS medium supplemented with kanamycin at 200 μg/ml.

Northern blot and RT-PCR analyses.

Total RNA (20 μg) was extracted from the cucumber after inoculation with the corresponding pathogen by the Phenol/SDS/LiCl method, separated on 1.2% formaldehyde/agarose gel, and subsequently transferred onto a nylon membrane. The specific expression pattern of the *CsGolS1* gene in the cucumber was measured by Northern hybridization using a DNA fragment of approximately 400 bp, which included the 3′ untranslated region of the cDNA. The DNA fragment was amplified by PCR using a synthetic forward primer (5′-gaagttgtcaac cacatc-3′) and an oligo (dT) primer as the reverse. The RNA blot was hybridized with the P^{32}-labeled cDNA probe and washed at a high stringency. Hybridization and P^{32}-labeling of cDNA were conducted in accordance with the standard procedures (Sambrook et al. 1989). For RT-PCR analysis, 500 ng of total RNA extracted from transgenic tobacco tissue by the Phenol/SDS/LiCl method was used along with the *CsGolS1* gene-specific primer (forward, 5′-TGCTGGTGAAGAAATGGT-3′; reverse, 5′-GCAGAAGGAAGCGTGATG-3′). The gene was amplified via 35 PCR cycles at 94°C for 39 s, 55°C for 1 min, and 72°C for 1 min.

Sugar analysis by HPLC.

Plant cell extracts were prepared from cucumber and transgenic tobacco tissues. Each plant tissue was frozen with liquid nitrogen, crushed, added to 20 ml of 50% methanol, and shaken for 1 h. The apparatus utilized for HPLC analysis was an LC 2000 series (Jasco, Tokyo) coupled with an evaporative light scattering detector (ELSD) 200 (SofTA, Broomfield, CO, U.S.A.). An ELSD nitrogen generator was employed as a source of nitrogen gas. Separation was conducted at 30°C on a reversed-phase Shim-pack CLC-NH_2 column (6 by 150 mm). ELSD conditions were optimized for maximum sensitivity: the spray chamber temperature was set at 35°C and the drift tube temperature was set to 60°C. 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.

Disease assessment on tobacco plants.

Five-week-old tobacco plants grown on soil in pots were inoculated with *B. cinerea* 4709 by spraying suspensions of 3.5×10^4 spores/ml of potato dextrose broth (PDB). Inoculated plants were kept in a growth chamber at 100% relative humidity for 24 h. Four to six days after inoculation, survival rate

was assessed by determining the number of symptomatic plants per pot. Six independent experiments were performed, with at least 50 plants per treatment. The fungus was grown on a potato dextrose agar (PDA) (Difco) plate for 2 weeks at 25°C in an incubator and the inoculum was then transferred to a one-half PDA plate, where it was further incubated for 1 week at 25°C in an incubator. To produce spores, the plates were again placed in an incubator for 2 days at 25°C with fluorescent illumination (approximately 2,000 Lux). Five-week-old tobacco plants cultivated on soil in pots were inoculated with *E. carotovora* SCC1 by spraying the bacterial suspension at a concentration of 1×10^8 CFU/ml onto the leaves. One to two days after inoculation, soft rot disease was rated by counting the number of symptomatic plants per pot. Six independent experiments were performed, with at least 50 plants per treatment.

Application of galactinol and disease assessment on tobacco plants grown in MS agar.

Galactinol dehydrate (Fluka 79544, purum grade, ≥98.0% [HPLC]) was purchased from Fluca Co. (Buchs, Sweden). In order to find the optimum concentration for application, sterile galactinol solution at concentrations of 0, 0.1, 0.5, 1, 5, and 10 mM were used to treat tobacco plants growing in sterilized growing medium. Tobacco seed were placed on MS agar supplemented with 1.5% sucrose in each well of a 12-well microtiter plate (SPL, Pocheon, Korea). Two weeks after the plants were seeded in the microtiter plate, 20 µl of 0.5 mM galactinol was applied to plant roots on MS agar in each well. At the same time, 20 µl of distilled water or 20 µl of another disaccharide (0.5 mM each of sucrose and lactose) was applied to plant roots in separated plates as a control or negative control, respectively. Five days after the treatment, plants were challenged with *B. cinerea* and *E. carotovoa*. In order to demonstrate induced resistance by galactinol treatment against the fungal pathogen, plants were inoculated with *B. cinerea* onto three leaves by pipetting 5 µl of the conidial suspensions of 2.5×10^5 spores/ml of PDB onto each leaf. The extent of symptoms (disease lesion area) caused by the fungus was measured 3 days after inoculation using the image inside software program version 2.32 (Ehwa Optical Co., Seoul, Korea). Three independent experiments were performed, with at least 100 plants per treatment. On the other hand, plants were inoculated with *E. carotovora* onto two leaves by pipetting 5 µl of the bacterial suspension of 8×10^8 CFU/ml onto each leaf. Two days after inoculation, soft rot disease was rated by counting the number of symptomatic plants in the microtiter plates (survival rate). Three independent experiments were performed, with at least 100 plants per treatment. A separate experiment was done to verify the effects on the survival rate by the bacterial pathogen. The galactinol-treated plants were challenged with *E. carotovora* onto two leaves by pipetting 5 µl of the bacterial suspension of 1×10^8 CFU/ml per each leaf.

Real-time RT-PCR.

Tobacco seed were placed on MS agar supplemented with 1.5% sucrose in each well of a 12-well microtiter plate (SPL Korea). Two weeks after the plants were seeded in the microtiter plate, 20 µl of 5 mM galactinol was applied to plant roots on MS agar in each well. Total RNA of the upper portion of the galactinol-treated plants was isolated using an RNeasy plant mini kit (cat. no.74904; Qiagen, Chatsworth, CA, U.S.A.) 0, 4, 8, 16, 24, and 48 h after the sugar treatment. For the detection of the specific RNA transcript, real-time quantification 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). The reaction mixture (25 µl) contained 500 ng of total RNA, 0.5 µM each primer, and appropriate amounts of enzymes and fluorescent dyes, in accordance with the manufacturer's instructions (Qiagen). For the control reaction, no RNA was added to the reaction mixture, resulting in no detectable fluorescence signal from the reaction. A Primer set for the 18S ribosomal RNA gene of tobacco plant (forward, 5′-AGGAATTGACGGAAGG GCA-3′; reverse, 5′-GTGCGGCCCAGAACATCTAAG-3′) was used as an internal standard. Data were analyzed using the software provided by the manufacture. Primers for defense-related genes used in this study are as follows: *PR1a* (forward, 5′-CCAATTGGCTGCAGATTGTA-3′; reverse, 5′-AGTTACG CCAAACCACCTGA-3′), *PR1b* (forward, 5′-ATACTTCTGC AGCGGGAAAC-3′; reverse, 5′-GATCACCGTAGGGACGTT GT-3′); *NtACS1* (forward, 5′-ATCCAAACGCTTCAATTTGC-3′; reverse, 5′-AATTGTTTCGTTGGCTCCAG-3′), and *NtACS2* (forward, 5′-TCCAAGAATGGGTGGTGAAT-3′; reverse, 5′-C TCCAGTTGCTCCTCCACTC-3′).

Drought and high-salinity stress treatments and subsequent damage assessment.

Both the CsGolS1 overexpressing transgenic and galactinol-treated wild-type tobacco plants were used in this experiment. In the experiment with CsGolS1 overexpressing transgenic tobacco, 5-week-old plants grown on soil in pots were soaked in 250 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 5-week-old plants grown on soil in pots until the plants showed visible wilting. Three independent experiments were performed with at least 50 plants per treatment. In the experiment with galactinol-treated wild-type tobacco, plant seed were placed on MS agar supplemented with 1.5% sucrose in each well of a 12-well microtiter plate. Two weeks after the plants were seeded in the microtiter plate, 20 µl of 0.5 mM galactinol was applied to the plant roots on MS agar in each well. At the same time, 20 µl of distilled water or of 0.5 mM sucrose was applied to plant roots in separated plates as a negative control and as a disaccharide control, respectively. Five days after the galactinol treatment, plants were then submitted to drought and high-salinity stresses. Plants grown in filter paper on MS agar plate were carefully removed and exposed to drought stress by placing them on an empty petri dish with half of the cover left open. Approximately 2 h after transposition, the survival rate (as assessed by the naked eye) and the weight of the stressed plant relative to the unstressed plant were quantitatively measured. Three independent experiments were performed, with at least 100 plants per treatment. To assess the relative salinity tolerance, the plants grown in filter paper on MS agar plates were carefully transferred into 250 mM NaCl solution and maintained for 5 days. The salt tolerance of the plants was estimated by measuring the survival rate and their total chlorophyll content. Three independent experiments were performed, with at least 100 plants per treatment.

Statistical analysis.

Data were analyzed by analysis of variance using JMP 4.0 software (SAS Institute, Cary, NC, U.S.A.). The significance of the observed effects was determined by Duncan's multiple range test ($P = 0.05$).

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