論　文（Original article）

Isolation of the drought- and salt-responsive galactinol synthase (GolS) gene from black poplar leaves and analysis of the transformants overexpressing GolS

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Abstract

Galactinol synthase (GolS), an enzyme synthesizing galactinol from *myo*-inositol and UDP-galactose, catalyzes the first step of the biosynthetic pathway of the raffinose family oligosaccharides (RFOs). A putative function of accumulated RFOs, galactinol and *myo*-inositol is compatible solute, but clear *in vivo* functions are uncertain. Six *GolS* genes were isolated from black poplar (*Populus nigra*) leaves. We characterized expression patterns of the *GolS* genes in response to either drought, salinity, cold stress or abscisic acid (ABA) and found the distinct *GolS* gene of which transcript level remarkably increased in response to drought- and salt-stress (*PnGolS2*). The amounts of leaf raffinose, galactinol and *myo*-inositol were significantly larger in the poplar transformants overexpressing *PnGolS2* (OXGolS) than those in non-transformants (NT). Leaf transpiration rate was significantly lower in the OXGolS plants than that in the NT due to the reduced stomatal conductance in the OXGolS. Leaf osmolality was unaffected by the overexpression of *PnGolS2* while the leaf water potential showed more negative values in the OXGolS plants than that in the NT plants. These results suggest that overexpressing the drought and salt-responsive *GolS* gene, *PnGolS2*, reduces hydraulic conductance in black poplar.

Key words: galactinol synthase, osmolality, poplar, stomatal conductance, transformant, water potential

1. Introduction

The accumulation and potential role of the raffinose family of oligosaccharides (RFOs) including raffinose and stachyose have been previously studied during seed maturity (Saravitz et al. 1987). Later, it has been also reported that RFOs accumulate in the vegetative organs and appear to play a prominent role in the stress tolerance of plants (Bachmann et al. 1994, Taji et al. 2002, Zhou et al. 2014).

Galactinol synthase (GolS), the enzyme that synthesizes galactinol from *myo*-inositol and UDP-galactose, can regulate RFO productions because GolS catalyzes the first step in the RFO biosynthetic pathway (Keller and Pharr 1996). Seven and nine genes encoding GolS proteins have been identified in *Arabidopsis thaliana* (*AtGolS1–7*; Taji et al. 2002) and in *Populus trichocarpa* (*PtrGolS1–9*; Zhou et al. 2014), respectively.

Abiotic stresses induce the expression of *GolS* genes in several plant species such as *A. thaliana* (Taji et al. 2002), tomato (Downie et al. 2003), coffee (dos Santos et al. 2011), rice (Saito and Yoshida 2011), and grapevine (Pillet et al. 2012). Recently, expression patterns of *GolS* genes have been characterized in *P. trichocarpa* subjected to stress treatments (Zhou et al. 2014). In *Arabidopsis*, Taji et al. (2002) studied stress responses of the three genes (*AtGolS1, AtGolS2,* and *AtGolS3*) and found that *AtGolS1* and *AtGolS2* expression were induced by drought or salinity, while *AtGolS3* expression was induced by low temperature. That study also demonstrated that overexpressing *AtGolS2* using the 35S promoter improved drought tolerance in *Arabidopsis*. Overexpressing *GolS* by genetic engineering has paved the way for improving the stress tolerance of plants (Sun et al. 2013, Zhuo et al. 2013, Himuro et al. 2014).

In the *Arabidopsis* transformant overexpressing the *GolS*, significant improvement in drought tolerance is likely due to reduced water consumption from the soil in the pot because the transformant had lower leaf transpiration rates (*E*) on leaf area basis than the non-transformant (Taji et al. 2002). On the other hand, *in vitro* studies show that RFOs and galactinol that

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have accumulated in the aerial organs function as compatible solutes (Santarius 1973) or scavengers of radical oxygen that is toxic to plants (Nishizawa et al. 2008). A putative function of accumulated RFOs, galactinol and *myo*-inositol is also osmoregulation (Ishibashi et al. 2011). However, water relations including water potentials and osmolyte concentrations of such transformants are not elucidated. The responses of stomatal conductance (g_s) and net CO_2 gas exchange rates (P_N) in such transformants are also unclear.

Black poplar (*Populus nigra*) is easily propagated by cuttings. In addition, studies on water relations such as measurements of water potential are more easily applicable to such tree species than to herbaceous species with rosette leaves, i.e., *Arabidopsis*. We previously constructed and analyzed the full-length cDNA libraries from *P. nigra* in order to study its stress physiology of *P. nigra* as well as for the functional analysis of its genes as a genetic resource (Nanjo et al. 2004, 2007).

The aims of this study are to isolate the *GolS* genes of which transcript abundance markedly response to drought or salinity stress in *P. nigra* and to reveal the function of GolS based on physiological analyses on the transformants overexpressing the *GolS* gene.

2. Materials and Methods

2.1 Plant materials and growth conditions

To isolate the *GolS* genes and characterize their expression patterns, a *P. nigra* clone was aseptically cultivated according to the method described in Nanjo et al. (2004). The tissue culture was grown at 25°C under cool white fluorescent light (16h/8h; day/night length). The photosynthetically active photon flux density was 40–60 μmol m^{-2} s^{-1} (LI-190SA, Li-Cor, MA).

For analyzing leaf gas exchange properties, sugar contents and water relations, poplar transformants (OXGolS) and non-transformants (NT) were hydroponically grown in an environmentally controlled growth chamber (Koito Electric Industries Ltd., Yokohama, Japan). The air temperature and relative humidity inside the chamber were set at 25°C and 75%, respectively. The photosynthetically active photon flux density measured at the top of these plants was 300 μmol m^{-2} s^{-1}. The lights (ceramic halide lamps) turned on at 6:00. Day/night length was 16/8 h. The plants were propagated by cuttings. Stems of 13 cm in length were cut out from OXGolS and NT plants and the base of the stems was submerged in tap water for 2 weeks. Next, the plant cuttings were transferred to Wagner pots (1/10,000-are, 1.4 L in volume) containing hydroponic culture (1/2,000 Hyponex 6-10-5, HYPONeX, Osaka, Japan). The culture medium was renewed twice every week. The plants were grown for 6 weeks after being transferred to Wagner pots.

2.2 Isolation and sequencing analysis of PnGolS cDNAs

Expressed sequence tags (ESTs) of *GolS* were queried using the BLAST+ program (Camacho et al. 2009) against the *P. nigra* full length cDNA libraries (Nanjo et al. 2004, 2007). The ESTs, PnFL2-078_L13, PnFL1-047_C08, PnFL2-032_B04, PnFL2-046_L21, PnFL1-093_N05, and PnFL1-055_M08 were re-sequenced. Each amino acid sequence was aligned using MUSCLE (Edgar 2004) in MEGA6 software (Tamura et al. 2013). Phylogenetic analysis was performed using the maximum likelihood method with the same software. Sequence identity and similarity were calculated by the FASTA program (Pearson and Lipman 1988) using GENETYX (ver.12 for Windows; GENETYX, Tokyo, Japan).

2.3 Stress and ABA treatments and RNA isolation

Stress and ABA treatments and RNA isolation were performed as described previously (Nanjo et al. 2004). The leaves of aseptically grown poplar were cut and subjected to dehydration, 400 mM NaCl, cold temperature (4°C) or 100 μM abscisic acid (ABA). For the dehydration treatment, the leaves were desiccated in 90 mm×20 mm petri dishes under dim light at an air temperature of 25°C and a relative humidity of 50–60%. For the cold treatment, the leaves were placed on a wet paper towel in a petri dish and then exposed to an air temperature of 4°C. For the NaCl treatment, the leaves were soaked in 50 mL aqueous solutions of 400 mM NaCl under dim light at 25°C. The leaves were treated for 1, 2, 5, 10 or 24 h and then frozen in liquid N_2 for RNA isolation. Each stress treatment was repeated three times. Quantitative real-time PCR (qPCR) was performed as described previously (Nishiguchi et al. 2012). The sequences of the DNA primers used for qPCR are shown in Table S1. The specificity of each amplified PCR product was confirmed by agarose gel electrophoresis and DNA sequencing. The relative mRNA level was normalized to the mRNA level of the ubiquitin gene.

2.4 Transformation of black poplar

To overexpress *PnGolS2* in *P. nigra*, the plasmid harboring the cDNA of *PnGolS2* was digested with *SfiI*. The excised cDNA fragment was inserted into the *SfiI*-digested pBE2113SF binary vector, which had the Cauliflower mosaic virus (CaMV) 35S promoter, the omega sequence of the Tobacco mosaic virus, the *SfiI* recognition sites, the nopaline synthase (NOS) terminator and the kanamycin-resistant *NPTII* gene (Ichikawa et al. 2006). The constructed vector was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. Transformation of *P. nigra* was performed as described previously (Mohri et al. 1996). Genomic DNA from poplar leaves was isolated using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). The *NPTII* gene introduced into the

transgenic poplar was confirmed by PCR using the specific DNA primers, NPT2U and NPT2R (Table S1), with the Quick Taq® HS DyeMix (Toyobo, Osaka, Japan). Conditions for the PCR reaction were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 68°C for 1.5 min, and 68°C for 5 min. An endogenous phosphoglycerate kinase gene (accession number AB018410) was amplified using the PnPGK_LP and PnPGK_RP primers (Table S1) as a positive control for PCR.

2.5 Sampling procedures for leaf gas exchange, water relation and sugar analyses

Fully expanded leaves positioned in the middle portion of the aerial parts were used for all measurements. Gas exchange and leaf water potential (ψ_L) experiments were performed between 9:00 and 14:00. For analyzing osmolality (π_L) and sugar contents, the leaves were also harvested between 9:00 and 14:00. These leaves were immediately frozen in liquid N_2 and were kept in a freezer (–80°C) until the measurements were taken. The number of sampled leaves was 15 for gas exchange measurements and it was 5–10 for ψ_L, π_L, and sugar content analyses. These leaves were taken from five plants.

2.6 Leaf gas exchange, osmolality and water potential

Leaf gas exchange parameters such as net CO_2 fixation rate (P_N), transpiration rate (E) and stomatal conductance (g_s) were measured using a portable infra-red CO_2/H_2O gas analyzer (LI-6400, Li-Cor). Light irradiated on the leaf surfaces was provided by a LED lamp (red + blue LED) equipped with the analyzer. The light intensity was adjusted to 400 μmol m^{-2} s^{-1}. The leaf temperature was set at 25°C and the leaf-to-air vapor pressure deficit was between 0.8 kPa and 1.2 kPa throughout the measurements. π_L of leaf sap was measured with an osmometer (Vapro 5520, Wescor, UT). Leaf sap was squeezed from the frozen leaf samples enclosed in a plastic bag using a pair of pliers. ψ_L was measured with a pressure chamber (Model 3000, Soil Moisture Equipment, CA).

2.7 Leaf sugar content

Frozen leaf samples were powdered in liquid N_2 using a mortar and pestle. Soluble sugars were extracted with 80% (v/v) ethanol including maltotetraitol as an internal standard at 90°C for 10 min in a water bath. After two additional extractions with 80% ethanol, the combined extracts were evaporated to dryness and dissolved in distilled water. The water-soluble fraction was extracted with chloroform to remove lipophilic materials and then passed through an ion exchange resin column (Dowex 50Wx8 H^+-form, The Dow Chemical, MI). Analysis of sugars was performed using an HPLC (LC-10A, Shimadzu, Tokyo, Japan) with a refractive index detector (RID-6A, Shimadzu) in a COSMOSIL Sugar-D column (4.6×250 mm, Nacalai Tesque, Kyoto, Japan) at 30°C. The eluent was 75% (v/v) acetonitrile in distilled water.

3. Results

3.1 Isolation of cDNAs encoding stress-induced *GolS* from black poplar

Many cDNAs homologous to *Arabidopsis GolS* (*AtGolS1, AtGolS 2,* and *AtGolS3*) were found in the *P. nigra* cDNA library. Their EST sequences were compared mutually to remove multiple redundant cDNAs. Therefore, six cDNAs encoding GolS were selected. PnFL2-078_L13, PnFL1-047_C08, PnFL2-032_B04, PnFL2-046_L21, PnFL1-093_N05, and PnFL1-055_M08. The predicted GolS proteins encoded by these six cDNAs consisted of 334 to 338 amino acid residues and could be divided into four groups (Table 1). Thus, the gene corresponding to each cDNA was newly named *PnGolS1* to *PnGolS4.2*. The PnGolS protein sequences were compared with the GolS proteins from *A. thaliana*, *P. trichocarpa* (Philippe et al. 2010, Zhou et al. 2014), and *Populus alba*×*grandidentata*

Table 1. Identity and similarity of the predicted amino acid sequences of galactinol synthase cDNAs isolated from *Populus nigra*.

Gene name	ID of cDNA	Predicted protein length (Amino acids)	% Identity (similarity) within the predicted PnGolSs					
			PnGolS1	PnGolS2	PnGolS3.1	PnGolS3.2	PnGolS4.1	PnGolS4.2
PnGolS1	PnFL2-078_L13	337	100 (100)	75 (93)	75 (92)	74 (91)	77 (93)	76 (93)
PnGolS2	PnFL1-047_C08	334		100 (100)	89 (99)	89 (99)	89 (99)	88 (98)
PnGolS3.1	PnFL2-032_B04	334			100 (100)	98 (99)	89 (97)	89 (97)
PnGolS3.2	PnFL2-046_L21	334				100 (100)	90 (97)	89 (97)
PnGolS4.1	PnFL1-093_N05	337					100 (100)	99 (99)
PnGolS4.2	PnFL1-055_M08	338						100 (100)

DDBJ accession numbers of the GolS cDNAs from *P. nigra* are as follows: PnFL2-078_L13, DB906648 and DB888707, PnFL1-047_C08, BP933107 and BP925488; PnFL2-032_B04, DB898289 and DB880105; PnFL2-046_L21, DB900909 and DB882796; PnFL1-093_N05, BP936598 and BP929152, PnFL1-055_M08, BP933724 and BP926146.

(Unda et al. 2012) (Fig. S1). Based on the alignment to these proteins, a phylogenetic tree was constructed (Fig. 1). PnGolS1 was in the same clade that includes PtrGolS8 (96% identity with PnGolS1, Table S2), PtrGol6 (92%), and AtGolS1 (82%). On the other hand, PnGolS2, PnGolS3.1, PnGolS3.2, PnGolS4.1 and PnGolS4.2 had low homology with PnGolS1 (Table 1) and formed a different clade. Among them, PnGolS2 showed high homology with PtrGolS3 (97%). PnGolS3 (PnGolS3.1 and PnGolS3.2) and PnGolS4 (PnGolS4.1 and PnGolS4.2) were highly homologous with PtrGolS1 and PtrGolS2, respectively (Table S2). PnGolS4.1 and PnGolS4.2 were also highly homologous with PaxgGolS2 from *P. alba*×*grandidentata* (98% and 97%, respectively).

3.2 Effects of environmental stress and ABA on *GolS* expression in black poplar

We used the *P. nigra* full-length cDNA libraries for the isolation of *GolS* cDNAs in this study. The cDNA libraries had been constructed from poplar plants subjected to environmental stress treatments such as dehydration, chilling,

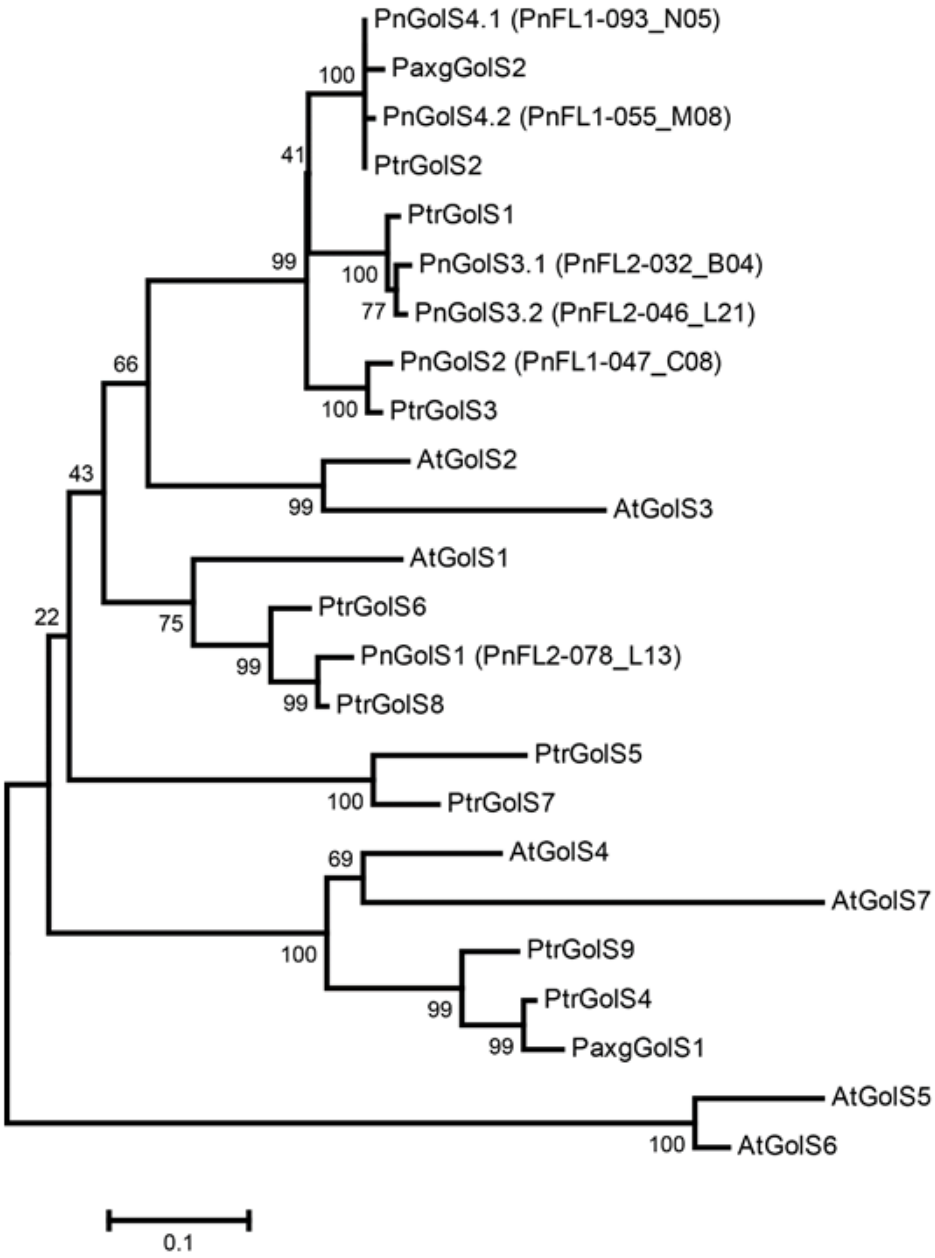

Fig. 1 Phylogenetic analysis of the predicted GolS proteins from *Populus nigra, P. trichocarpa, P. alba* × *grandidentata*, and *Arabidopsis thaliana*.

The names of the GolS proteins from *P. trichocarpa* are based on the report by Zhou et al. (2014). The phylogenetic tree was constructed with the maximum likelihood method. Bootstrap values are based on 1000 replicates. The bar corresponds to 0.1 amino acid substitutions per site.

high concentration of NaCl, heat, ABA, and hydrogen peroxide (Nanjo et al. 2004, 2007). The gene expression of *AtGolS1*, *AtGolS2*, and *AtGolS3* is induced by environmental stress (Taji et al. 2002). Accordingly, the gene expression of the isolated *PnGolS* genes is expected to change in response to environmental stress. To confirm this hypothesis, we measured the mRNA levels of each *PnGolS* gene in leaves under drought, NaCl, cold, or ABA treatment using qPCR.

The expression of *PnGolS1* was increased 10- to 100-fold by drought, NaCl, cold, and ABA treatment (Fig. 2). The rapid induction of *PnGolS1* expression could be detected at 1 h after each treatment. *PnGolS2* was remarkably induced by drought- and NaCl-stress treatment (Fig. 2). The mRNA abundance of *PnGolS2* reached approximately 13,000-fold at 10 h after drought treatment and approximately 5,700-fold at 10 h after NaCl treatment. Cold treatment increased the expression of *PnGolS2* by approximately 370-fold at 24 h after treatment. *PnGolS3.1* and *PnGolS3.2* were also induced by drought and NaCl (Fig. 2). However, the increases in the mRNA abundance of *PnGolS3.1* and *PnGol3.2* (10- to 30-fold) were remarkably

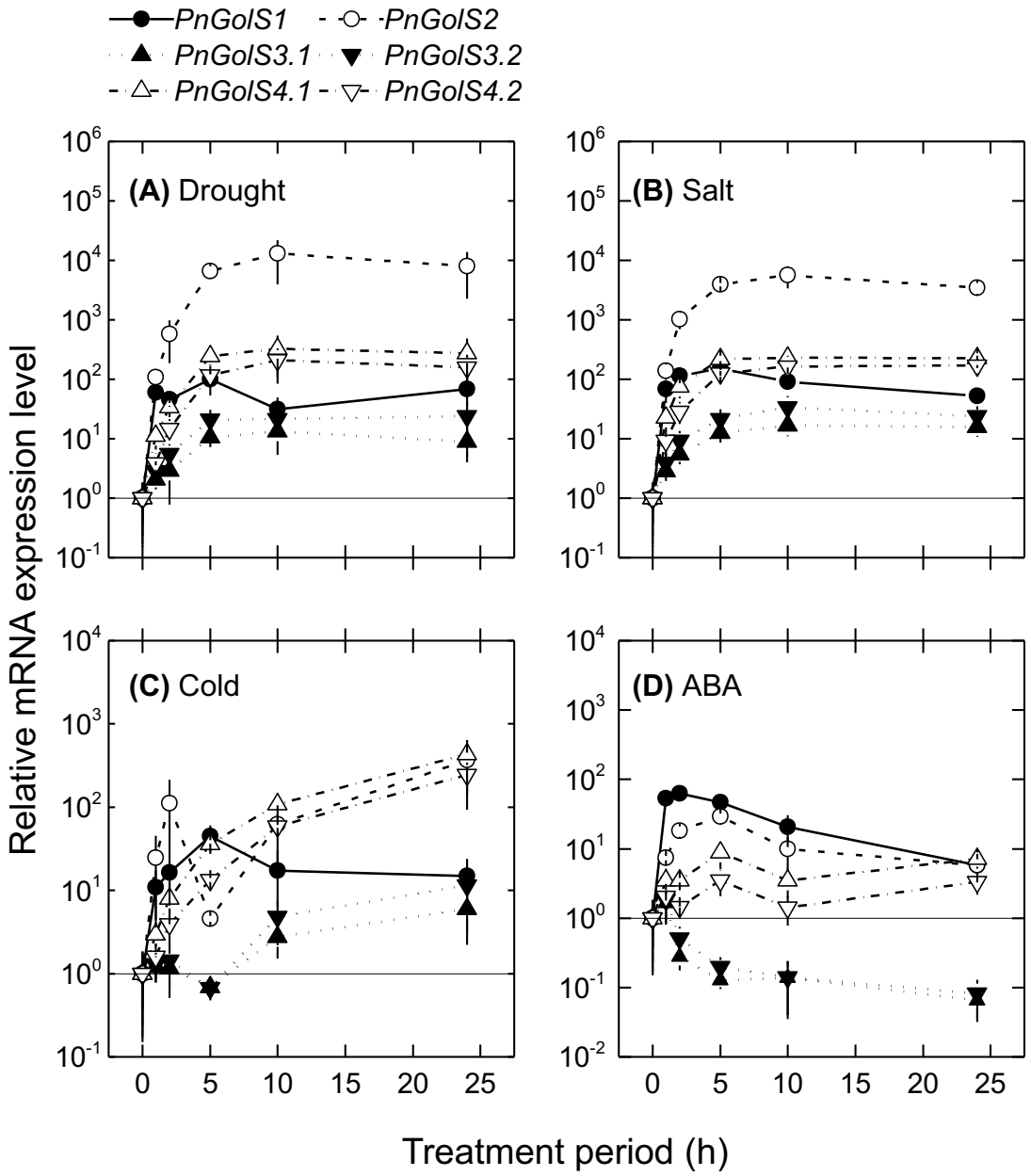

Fig. 2 The effect of drought, salt, cold, and abscisic acid (ABA) treatments on the gene expression of *PnGolS* in the lamina of *Populus nigra*.

Leaves of tissue-cultured *P. nigra* were cut and subsequently subjected to (A) dehydration, (B) 400 mM NaCl, (C) 4°C or (D) 100 μM ABA treatment. The mRNA level of samples subjected to treatments was normalized to that of the non-treated sample (0 h). Error bars represent the SD (n = 3 experiments).

lower than those of the *PnGolS2*. Similarly, the expression of *PnGolS4.1* and *PnGolS4.2* were induced by drought, NaCl, and cold treatments (Fig. 2): particularly, these genes responded to cold (by 440-fold and 240-fold increased expression, respectively). *PaxgGolS2* in *P. alba*×*grandidentata* (Unda et al. 2012) and *PtrGolS2* in *P. trichocarpa* (Zhou et al. 2014) are also *GolS* genes that show a remarkable upregulation in the expression in response to cold. The predicted amino acid sequences of the *PaxgGolS2* and *PtrGolS2* were structurally similar to those of *PnGolS4.1* and *PnGolS4.2* (Fig. 1). ABA treatment elevated the gene expression of *PnGolS1*, but its effect on the gene expression of the other *PnGolSs* was very small.

3.3 Generation of poplar transformants overexpressing *GolS*

We generated poplar transformants overexpressing *PnGolS2* (OXGolS) because *PnGolS2* showed the highest expression under drought and salinity conditions among the investigated *PnGolS* genes (Fig. 2). The cDNA of *PnGolS2* was fused downstream of the CaMV 35S promoter in the binary vector pBE2113SF (Fig. 3A). *P. nigra* was transformed with *Agrobacterium* harboring pBE2113SF::PnGolS2. Three kanamycin-resistant poplar plants were regenerated. PCR analysis confirmed that three lines of the poplar plants had the *NPTII* gene but only a single line was successfully sustained (Fig. S2). The expression level of *PnGolS2* in the leaves of OXGolS plants was approximately 280,000-fold higher than that in NT plants (Fig. 3B). This result indicates that the introduced *PnGolS2* cDNA was constitutively overexpressed in the OXGolS group. Nishizawa et al (2008) reported that no morphological difference was found between the *Arabidopsis* transformants overexpressing *GolS* and the wild type. As was the case for *Arabidopsis*, the morphological appearance was unaffected by the *GolS* overexpression in poplar (Fig. S3).

3.4 Leaf gas exchange, sugar content and water relations in poplar overexpressing *GolS*

Taji et al. (2002) reported that overexpressing *GolS* reduced the leaf transpiration rate (*E*) in *A. thaliana*. As was the case for *Arabidopsis*, *E* significantly decreased in the *P. nigra* transformants overexpressing *GolS* (Table 2). The decrease in *E* was largely due to the decrease in stomatal conductance (g_s). The significant reduction of g_s was observed while the net CO_2 exchange rate (P_N) was similar between NT and OXGolS plants. From these results, the calculated water-use efficiency (WUE) was significantly higher in the OXGolS plants than in the NTs (Table 2).

Overexpressing *PnGolS2* significantly increased the leaf galactinol, raffinose and *myo*-inositol contents (Table 2). Stachyose content was lower than the detection limit in both transformants and NT. Again, the amount of galactinol, a GolS reaction product, was significantly enhanced by *GolS* overexpression. This strongly suggests that the transgene was active in the transformants. The differences in osmolality (π_L) between NT and OXGolS leaves were unclear although significant increases in the galactinol, raffinose, and *myo*-inositol content were observed for the OXGolS group (Table 2). Water potential (ψ_L) showed more negative values in the OXGolS than in the NT (Table 2).

4. Discussion

4.1 Functional differentiation in gene expression between multiple *GolS* genes in black poplar leaves

The six isolated cDNAs encoding *GolS* genes were structurally compared with each other and with *GolS* genes from other *Populus* species and *A. thaliana*, after which we named the putative genes corresponding to each cDNA, *PnGolS1, PnGolS2, PnGolS3.1, PnGolS3.2, PnGolS4.1*, and

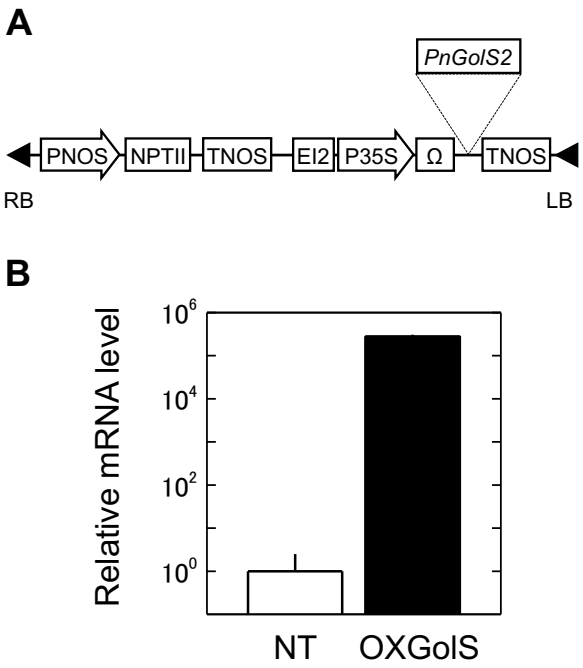

Fig. 3 Overexpression of *PnGolS2* in Populus nigra. (A) Construction of the pBE2113SF::PnGolS2 binary vector.

The *PnGolS2* cDNA was inserted downstream of the omega sequence. PNOS, promoter of nopaline synthase; NPTII, neomycin phosphotransferase; TNOS, terminator of nopaline synthase; EI2, two tandem repeats of 5'-upstream sequences of Cauliflower mosaic virus (CaMV) 35S promoter; P35S, CaMV 35S promoter; Ω, Tobacco mosaic virus omega sequence. (B) Expression of *PnGolS2* in non-transformant (NT) and transformants (OXGolS). The mRNA level of *PnGolS2* was normalized to that of the NT group. Error bars represent the SD (n = 3 experiments).

PnGolS4.2, respectively. There was a slight difference in the amino acid sequence between PnGolS3.1 and PnGolS3.2 and between PnGolS4.1 and PnGolS4.2. Therefore, PnGolS3.1 and PnGolS4.1 may be allelic variants of PnGolS3.2 and PnGolS4.2, respectively. Putative *GolS* alleles with amino acid substitutions have been reported for *P. trichocarpa* and *P. trichocarpa*×*deltoides* (Philippe et al. 2010).

Only *PnGolS1* expression was induced by ABA, while the other *PnGolS* genes were not significantly induced (Fig. 2). This result suggests that *PnGolS1* is induced in an ABA-dependent pathway. *PnGolS1* showed a highly homologous to *PtrGolS8* and *PtrGolS6* in *P. trichocarpa* (Fig. 1 and Table S2). On the other hand, a significant elevated expression by ABA was not observed in *PtrGolS6* or *PtrGolS8* (Zhou et al. 2014). Zhou et al. did not follow the expression patterns of *GolS* over time in response to exogenous ABA, which may explain why a clear ABA-dependent was not observed.

The expression level of *PnGolS2* peaked at 10 h after drought treatment, and then decreased by 24 h, and this expression pattern showed the same trend under NaCl treatment (Fig. 2). In a study by Zhou et al. (2014), PtrGolS3 from *P. trichocarpa* was structurally similar to PnGolS2 in the black poplar (Fig. 1) and also exhibited a strong upregulation in gene expression at 10 h after drought treatment and sharply declined by 24 h.

The response of *PnGolS2* to drought and salt stresses was distinct from that of the other *GolS* genes, suggesting that *PnGolS2* significantly contributes to stress responsive galactinol synthesis. In contrast, the induction of *PnGolS3.1* and *PnGolS3.2* by stress treatments was significantly lower than that of the other *PnGolS* genes. In addition, *PnGolS4.1* and *PnGolS4.2* were strongly induced by cold treatment. Thus, we clearly show that the expression of multiple *GolS* genes are functionally differentiated in black poplar leaves.

4.2 Overexpressing *GolS* reduces stomatal conductance in the poplar

Data on the g_s and P_N of *Arabidopsis* overexpressing *GolS* were not provided in the study by Taji et al. (2002). In this study, we clearly show that the decrease in *E* was due to the decrease in g_s in the GolS-overexpressed poplars (Table 2). It also appeared that leaf yellowing symptoms due to water deficient was delayed in the potted transformants compared with NT (Fig. S4), which might be related with the reduced g_s of the transformants.

A putative function of accumulated galactinol, *myo*-

Table 2. Leaf gas exchange properties, sugar contents and water relations of non-transformants (NT) and transformants overexpressing *PnGolS2* (OXGolS) in *Populus nigra*.

		NT	OXGolS	*p*	*n*	% Change
Gas exchange properties						
Net CO_2 fixation rate (P_N)	μ mol CO_2 m^{-2} s^{-1}	9.8 ± 2.2	9.2 ± 2.3	ns	15	−6
Transpiration rate (*E*)	mmol m^{-2} s^{-1}	5.5 ± 0.4	4.1 ± 0.5	***	15	−25
Stomatal conductance (g_s)	mol m^{-2} s^{-1}	0.93 ± 0.12	0.50 ± 0.11	***	15	−46
WUE (= P_N / g_s)	μ mol mol^{-1}	11 ± 1.8	18 ± 3.8	***	15	+63
Sugar content						
Galactinol	μ mol gFW^{-1}	0.12 ± 0.26	1.7 ± 0.58	***	5	+1316
Raffinose	μ mol gFW^{-1}	0.07 ± 0.10	0.3 ± 0.22	*	5	+329
myo-inositol	μ mol gFW^{-1}	11 ± 2	21 ± 2	***	5	+91
Fructose	μ mol gFW^{-1}	31 ± 13	25 ± 3	ns	5	−19
Glucose	μ mol gFW^{-1}	39 ± 16	40 ± 12	ns	5	+3
Sucrose	μ mol gFW^{-1}	12 ± 7	14 ± 7	ns	5	+17
Water relations						
Osmolality (π_L)	mmol kg^{-1}	655 ± 64	640 ± 48	ns	10	−2
Water potential (ψ_L)	MPa	−0.67 ± 0.9	−0.85 ± 1.6	**	10	+27

WUE is instantaneous water-use efficiency. Stachyose was not detected. The percent change by *GolS* overexpression was calculated as: (N–T)/N×100, where N and T are the mean values for NT and for OXGolS, respectively. FW, fresh weight. The values are mean ± SD. *p*-values for student ***t***-tests are shown: *, <0.05; **, <0.01; ***, <0.001. ns, not significant. *n* is the number of sample leaves.

inositol, and RFOs is osmoregulation (Ishibashi et al. 2011). However, π_L was unaffected by *GolS* overexpression (Table 2), meaning that the increases in leaf galactinol, raffinose and *myo*-inositol content by the *GolS* overexpression were not enough to increase the osmolality.

During a steady-state condition, ψ_L is expressed as a function of E (Kaufmann 1976) as follows:

$$\psi_L = \psi_S - \frac{E}{K_{SL}} \quad (1)$$

where ψ_S and K_{SL} are soil water potential and hydraulic conductance from the soil to the leaf, respectively. Equation (1) indicates that ψ_L linearly increases (i.e. shifting to positive values) as E decreases when K_{SL} is assumed to be constant. However, ψ_L showed more negative values in OXGolS than in NT although OXGolS had a lowered E (Table 2), implying that K_{SL} is different between OXGolS and NT plants.

Equation (1) can be rewritten as follows:

$$K_{SL} = \frac{E}{(\psi_S - \psi_L)} \quad (2)$$

We estimated K_{SL} from the mean values of each measured E and ψ_L using equation (2), based on the assumption that ψ_S is zero and is not different between the transformants and NT groups. The estimated K_{SL} values were 8.3 and 4.8 mmol m^{-2} s^{-1} MPa^{-1} for NT and OXGolS, respectively. Thus, the estimated K_{SL} value was considerably lower in the OXGolS plants than that in the NT plants. Previous studies suggest that K_{SL} controls g_s via unidentified signals (hydraulic signal) in contrast to the well-known chemical signaling by which ABA controls g_s (Comstock 2002, Hacke 2014). Lowered hydraulic conductance may explain the reduced g_s following *GolS* overexpression. Further studies need to confirm this hypothesis by a direct measurement of K_{SL} and to clear underlying mechanisms how the *GolS* product controls K_{SL}.

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Table S1. The DNA primers used in this study.

Table S2. Relationship of galactinol synthase (GolS) from *Populus nigra* with those from *P. trichocarpa*.

Fig. S1 Comparison of the predicted amino acid sequence of galactinol synthase (GolS) from *Populus nigra* with those of other GolSs from *P. trichocarpa, P. alba* × *grandidentata,* and *Arabidopsis thaliana*. A putative serine phosphorylation site is indicated by an asterisk and the characteristic hydrophobic pentapeptide (APSAA) at the C-terminus is shown by a black bar.

Fig. S2 PCR confirmation of transgene integration. The neomycin phosphotransferase gene (*NPTII*) was amplified from only the genomic DNA of a *PnGolS2*-overexpressed poplar (OXGolS), whereas a non-transformant (NT) did not have the *NPTII* gene. A phosphoglycerate kinase gene (*PGK*, accession number AB018410) was used as a positive control for PCR. M, Lambda DNA/HindIII marker.

Fig. S3 Photographs of (A) non-transformants and (B) transformants overexpressing *PnGolS2* in *Populus nigra*. The photographs were taken 8 weeks after transfer to the Wagner-pots. The scale bar on the right bottom corner equals 10 cm in length.

Fig. S4 Photographs of the transformants overexpressing *PnGolS2* in *Populus nigra* (OXGolS) and the non-transformants (NT) (A) before and (B) on 14th day after withholding water supply. Aseptically cultivated clones were transplanted in a 1/10000 Wagner pot filled with 40 g vermiculite (in dry weight per pot). The plants were grown during about two months under an environmentally-controlled growth chamber before withholding water supply. The photosynthetically active photon flux density over the plants was 300–500 μmol m^{-2} s^{-1}. The scale bar on the right bottom corner equals 10 cm in length.

セイヨウハコヤナギの葉における乾燥・塩ストレス応答性ガラクチノール合成酵素（Galactinol synthase, GolS）遺伝子の単離と GolS 過剰発現ポプラの解析

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要旨

ガラクチノール合成酵素（Galactinol synthase, GolS）は *myo*-イノシトールと UDP-ガラクトースからガラクチノールを合成する酵素であり、ラフィノース族オリゴ糖類（Raffinose family oligosaccharide, RFO）合成経路の初発反応を触媒する。植物体に蓄積する RFO、ガラクチノール、*myo*-イノシトールは適合溶質としての機能などが示唆されているが、生体内でのはっきりとした役割は明らかではない。我々はセイヨウハコヤナギ（*Populus nigra*）の葉から 6 種類の *GolS* 遺伝子を単離し、乾燥や塩ストレスに応答して発現量が顕著に増加する *GolS* 遺伝子（*PnGolS2*）を見出した。*PnGolS2* を過剰発現した形質転換セイヨウハコヤナギ（OXGolS）の葉に含まれるラフィノース、ガラクチノール、*myo*-イノシトールの含量は、非形質転換体（non-transformant, NT）よりも顕著に増加した。また、OXGolS の気孔コンダクタンスは NT と比べると低下し、その結果、OXGolS の葉の蒸散速度は大きく減少していた。*PnGolS2* を過剰発現しても葉の浸透圧に大きな影響はなかったが、一方で、過剰発現によって葉の水ポテンシャルは大きく低下した。これらの結果は、乾燥・塩ストレス応答性 *GolS* 遺伝子である *PnGolS2* を過剰発現すると、セイヨウハコヤナギの通導コンダクタンスを低下させることを示唆している。

キーワード：ガラクチノール合成酵素、浸透圧、ポプラ、気孔コンダクタンス、形質転換体、水ポテンシャル

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