

High concentrations of sucrose induce overwintering bud formation in gentian plantlets cultured in vitro

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Abstract Overwintering buds (OWBs) are dormant organs produced by perennial plants. After flowering, Japanese gentians (*Gentiana triflora*, *G. scabra* and their hybrids) produce OWBs in autumn and go dormant to survive winter. Although dormant OWBs have been reported to have strong cold and freezing tolerances, no details about mechanisms and regulation are known. We attempted to produce in vitro OWBs (IOWBs) from gentian plantlets to facilitate the study of OWBs. We found that high-sucrose (6%) culturing media induced IOWB formation. IOWBs showed an enhanced freezing tolerance together with high levels of proteins and transcripts known to specifically accumulate in OWBs. Furthermore, IOWBs showed endodormancy release and bud break in the same manner as OWBs. Bud breaking in OWBs and IOWBs was promoted by red light and inhibited by far-red light. These results indicated that IOWBs possess similar physiological characteristics as OWBs, at least regarding stress tolerance and dormancy. Therefore, our artificial IOWB induction technique will be useful for the study of physiological mechanisms in field-grown OWBs.

Key words: Dormancy, gentian, high sucrose culture, overwintering buds.

Gentians are herbaceous perennial plants distributed in alpine regions. There are over 400 species worldwide; *Gentiana triflora* and *G. scabra* are native to Japan. Japanese gentians produce blue, white, or pink flowers from early summer to late autumn. After flowering, they develop crown buds called overwintering buds (OWBs) which survive the cold season. From the middle of the 1950's, various cultivars and lines were created by hybridization using these wild gentians. In recent years, F₁ hybrid cultivars exhibiting heterosis have been grown commercially as cut or pot flowers. However, it is necessary to maintain the genetic purity of the parental lines to obtain F₁ plants of constant phenotypes. To this end, in vitro clonal culture of gentian plantlets has been developed (Hosokawa et al. 1996). In vitro culture techniques are widely used as tools for the mass propagation of ornamental plants such as roses, carnations, chrysanthemums, and cyclamens (Kantia and Kothari 2002; Ma et al. 1996; Malgorzata et al. 2007; Winkelmann 2010). Because in vitro clones have same genetic backgrounds and similar phenotypes, this biotechnology facilitates and accelerates the generation of new cultivars. In gentian, unfortunately, many cultivars and lines are difficult to grow under in vitro

culture conditions. The principal reason is that these varieties and lines bloom during cultivation, resulting in death. Therefore, inhibition of flowering is the most important aim in culturing these lines. Recently, we reported that gentian orthologs of *FLOWERING LOCUS T* (*GtFT1* and *GtFT2*) and *TERMINAL FLOWER1* (*GtTFL1*) contributed to the regulation of flowering time in field-grown *G. triflora* plants (Imamura et al. 2011). Prior to flowering, the expression levels of *GtFT1* and *GtFT2* increased, whereas that of *GtTFL1* decreased. Furthermore, up-regulation of *GtFTs* or down-regulation of *GtTFL1* promoted floral initiation. Flowering was accelerated by various environmental and endogenous factors, including photoperiod, light quality, temperature, gibberellins, and sucrose (Srikanth and Schmid 2011). However, since the triggering factor of gentian flowering remains unknown, techniques for flowering inhibition are currently not available. To repress flowering in gentian plantlets, we focused on in vitro OWBs (IOWBs). IOWBs are OWB-like buds rarely produced in a limited number of varieties and lines of gentian (Takahashi et al. 2012). Since IOWBs cannot bloom and grow much slower than plantlets, they are the preferred material for long-term maintenance. Recently,

Abbreviations: IOWBs, in vitro overwintering buds; OWBs, overwintering buds.

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Takahashi et al. (2012) reported that phosphorus (P) deficiency during cultivation induced IOWB formation even in cultivars and lines that do not produce IOWBs under normal culture conditions. The IOWBs produced under P deficiency showed almost no growth but started to grow again after transfer to sufficient P, indicating that the morphological transition from plantlets to IOWBs might be a strategy to survive the duration of stressful conditions, which would be a similar mechanism as OWBs produced in field-grown gentian plants. Although the artificial formation of IOWBs may facilitate research into the ecology and physiology of OWBs, the removal of essential nutrients is not practicable in the context of long-term maintenance. Furthermore, it is unclear at this stage whether IOWBs resemble OWBs regarding stress tolerance and dormancy. Dormancy in vegetative organs such as OWB, tuber and crown is a programmed strategy to survive environmental extremes. Dormancy can be divided into three stages: para-, endo- and ecodormancy (Horvath et al. 2003). In paradormancy, bud growth is inhibited by signals produced in other parts of the growing plant. Endodormancy also is referred to as innate dormancy: buds will not grow even if they experience the appropriate environmental conditions for growth. Ecodormancy is the last dormant state and is regulated by environmental conditions. Endodormancy release is the transition from endodormant to ecodormancy, requiring extended periods of cold temperatures. A previous observation showed that in gentian OWB, an exposure of approximately 700 h to temperatures of 5°C or lower is required to release endodormancy (Yoshiike 1992). However, relationship between cold exposure and dormant stages in IOWB is still unknown.

In this study, we found that gentian plantlets cultured on MS medium containing high concentrations of sucrose frequently induced IOWB formation. Comparing IOWBs and OWBs, we found that IOWBs accumulated several transcripts and proteins that usually are accumulated specifically in OWBs. Moreover, IOWBs exhibited enhanced freezing stress tolerance compared to other organs, and showed endodormancy release and bud break similar to OWBs. From these results, we propose that IOWBs possess similar characteristics as OWBs of field-grown gentian plants.

Materials and methods

Plant materials, growth conditions and high sucrose treatment

Gentiana triflora cv. 'SpB' grown in an agricultural field in Iwate Biotechnology Research Center was used. Plantlets of *G. triflora* cv. 'Ihatovo' and 'Maciry' were cultured in vitro on a solid Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.2% (w/v) gellan gum at 22°C under a 16/8 h

light/dark photoperiod. To test the effects of different light conditions, plantlets were cultured with red light at 660 nm and far-red light at 735 nm peak wavelength from light-emitting photodiodes (red; ISL-150×150-RR, far-red; ISL-150×150-FF, CCS lic., Kyoto, Japan). A fluorescent lamp (FL40ss/W/37, Panasonic, Osaka, Japan) was used as the white light source. All light intensities were measured with a SUN ILLUMIIND SLX-1330 (Sansyo, Tokyo, Japan) and set to 40 μmol m⁻² s⁻¹. For subculture, individual shoots were separated from proliferating cultures before transfer to fresh MS medium every three months. For high sucrose treatment, plantlets were cultured in vitro on solid MS medium containing 6% (w/v) sucrose and 0.2% (w/v) gellan gum at 22°C under a 16/8 h light/dark photoperiod. For further analysis, harvested plants and plantlets were frozen in liquid nitrogen, freeze-dried, and stored at -20°C until use.

Measurement of sugar concentration in IOWBs

The extraction of metabolites was performed according to Takahashi et al. (2009). Ten mg of freeze-dried samples were powdered with a Micro Smash M100 (Tomy, Tokyo, Japan), homogenized with 200 μl 50% (v/v) methanol for 10 min, and the homogenate was centrifuged at 20,000×g for 5 min. The supernatant was filtered through a 3-kDa-cutoff filter (Millipore, Bedford, MA) and the filtrates were used for further analysis. Sugars were analyzed on an Agilent 6510 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) with a Hypercarb column (4.6×100 mm, 5 μm, Thermo Scientific, Vantaa, Finland); column temperature was maintained at 60°C. The mobile phase (A: water; B: acetonitrile) was pumped at a flow rate of 0.3 ml/min with a gradient program as follows: 0–8% B in 2 min; 8% B for 2 min; 8–11% B in 10 min; 11–100% B in 2 min; 100% B for 10 min. In TOF-MS, the fragmentor, skimmer, and OCT RFV voltages were set at 100, 60, and 300 V, respectively. Data were acquired in the negative ion mode and quantitative accuracy was estimated with known concentrations of reference standards.

Protein extraction

Two grams (fresh weight) of gentian tissues were ground into powder in liquid nitrogen. The powder was suspended in 1 ml extraction buffer (6 M urea, 5% β-mercaptoethanol, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM phenylmethylsulfonyl fluoride (PMSF)), vortexed for 5 min at 4°C, and centrifuged for 10 min at 20,000×g at 4°C. To precipitate soluble protein, 1/10 volume of trichloroacetic acid (TCA) were added to the supernatant, incubated for 15 min at 4°C, and centrifuged for 15 min at 15,000×g at 4°C. The supernatant was discarded and the pellet was washed in 1 ml ice-cold ethanol, and then centrifuged for 10 min at 15,000×g at 4°C. This washing step was repeated two more times. After washing, the pellets were dried in air and stored at -20°C until analysis. Pellets were solubilized in 400 μl sample buffer containing 8 M urea, 5% β-mercaptoethanol, 2% CHAPS, 0.5% IPG buffer pH 4–7 (GE Healthcare,

Buckinghamshire, UK), centrifuged for 1 min at $10,000\times g$ at 4°C . Protein concentration was quantified immediately after extraction using Bradford's method with bovine serum albumin as a standard (Sigma-Aldrich, St. Louis, MO).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The first dimension isoelectric focusing (IEF) was performed using a Cool Phore Star IPG-IEF Type-P (Anatech, Tokyo, Japan) and a 13 cm Immobiline DryStrip (GE Healthcare) with a 4–7 pH gradient. IPG strips were covered with mineral oil to prevent dehydration and were actively rehydrated at 20°C for 14 h. A 1.2 mg sample of protein was focused at 500 V for 3 h, 500 to 3,500 V for 4.5 h (linear ramp) and 3,500 V for 8 h. IPG strip equilibration took place in equilibration buffer (50 mM Tris-HCl [pH 6.8], 6 M urea, 1% sodium dodecyl sulfate (SDS), 30% glycerol, 0.0025% bromophenol blue) containing 3% β -mercaptoethanol for 30 min, followed by equilibration buffer containing 4.5% iodoacetamide for 20 min. IPG strips were then loaded onto 15% SDS-PAGE gels and run at 10 mA/gel until the dye from the agarose sealing gel had migrated into the resolving gel and then at 40 mA/gel until the dye front had run off the gel. Gel was stained with Coomassie Brilliant Blue. Protein profiles were obtained using five independent biological replicates per treatment. After electrophoresis, the gels were scanned using an ImageQuant LAS 4000 (GE Healthcare).

Protein digestion and identification

Selected spots were excised, digested with trypsin using the In-Gel Tryptic Digestion Kit (Thermo Scientific), and was purified using Pierce C-18 Spin Columns (Thermo Scientific). Enzyme digests were applied for nano LC-MS/MS analysis. Digested peptides were eluted from a trap column with 0.1% formic acid in acetonitrile and separated with a Magic C18 AQ nano column (MICHROM Bioresources, Auburn, CA). The eluting solution was linearly changed from 5 to 45% acetonitrile at a flow rate of 500 nl min^{-1} . The mass spectrometer used was an LTQ Orbitrap XL (Thermo Scientific) operated with the Xcalibur software (Ver. 2.0.7, Thermo Scientific). Peptides were identified using a MASCOT MS/MS ion search (<http://www.matrixscience.com/home.html>) in error tolerance mode using gentian EST database (data not shown).

Real-time RT-PCR analysis

Total RNA was extracted from organs of *G. triflora* with the RNAs-ici!-P (Rizo Inc., Tsukuba, Japan), and treated with RNase-free DNase I (Wako, Osaka, Japan) to eliminate genomic DNA. First-strand cDNA was synthesized from 500 ng total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA) with random primers according to the manufacturer's instructions. Quantitative real-time RT-PCR was carried out using StepOnePlus (Applied Biosystems) with the KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems, Woburn, MA). The amplification conditions were 95°C for 20 s, followed by 40 cycles of 3 s at 95°C and

30 s at 60°C , and plate reading after each cycle. The data was analyzed using the StepOne software (Applied Biosystems). Primers used for real-time RT-PCR for *GtDHN1* were: forward primer (5'-CAA AGG ACA CGG CCA AGG T-3') and reverse primer (5'-GAC CGT CAT CCT CTG ACG AAC T-3'), for *GtDHN2*: forward primer (5'-TCA AAA AAC ACG TCG GAG GAT-3') and reverse primer (5'-CCC AGG AGT AGT AGC AGCTCCAT-3'). The *G. triflora ubiquitin* gene was used as normalization control; primers used were forward (5'-GGA AGC AGT TGG AAG ATG GC-3') and reverse (5'-GGC CAA AGT TCG TCC GTC CTC-3').

Evaluation of freezing stress tolerances

Freezing stress tolerance was evaluated by analysis of electrolyte leakage. To evaluate freezing tolerance, gentian tissues were exposed to -8°C for 3 h. Determination of electrolyte leakage was performed according to the method of Imamura et al. (2013). Approximately 20 mg samples were washed with deionized water, immersed in 1 ml deionized water for 16 h, and the initial electrical conductivity was measured using a B-173 detector (Horiba, Kyoto, Japan). The samples were then boiled for 15 min and the final conductivity was measured after cooling to room temperature. The electrolyte leakage is defined operationally as the final conductivity expressed as percentage of the initial conductivity.

Evaluation of dormant state in IOWB and OWB

To evaluate the dormant state, in plantlets, IOWBs were exposed to cold temperature (4°C). Five weeks after the exposure, IOWBs were incubated at 22°C for 3 weeks to induce bud break. In plants, OWBs in October and in March were harvested and incubated at 22°C for 3 weeks to induce bud break. By definition, endodormant OWBs and IOWBs did not sprout, whereas ecodormant ones did.

Results and discussion

High concentration of sucrose induce IOWB formation

Previously, we found that P deficiency induced IOWBs in gentian plantlets (Takahashi et al. 2012). However, this method was not suitable for long-term maintenance, because all other organs expect for the IOWBs died. Therefore, we attempted to develop alternative methods to induce IOWB formation. In plantlet culture, sugars are one of the most important factors as energy sources and regulators of morphogenesis (Roldan et al. 1999). We compared sugar contents between shoots and IOWBs: the concentrations of glucose and fructose were lower in IOWBs than in shoots, whereas sucrose accumulated in IOWBs to levels that were approximately threefold higher than in shoots (Figure 1). Therefore, we focused on sucrose. Two gentian cultivars, 'Thatovo' and 'Maciry', were cultured under high sucrose conditions (MS medium containing 6% sucrose), to evaluate the effects

of exogenous sucrose. 'Ihatovo' readily forms IOWBs under standard conditions (MS medium containing 3% sucrose), whereas 'Maciry' does not (Figure 2). The rates of IOWB formation in 'Ihatovo' and 'Maciry' were 2.5- and 23-fold higher, respectively, under high sucrose compared to standard conditions (Figure 2C and 2D). Thus, high sucrose levels induce IOWBs in gentian plantlets. Although the mechanism of IOWB formation is still unknown, it possibly is related to stress-survival strategies. Previous reports showed that in vitro bud formation of temperate grass species could be induced by high concentrations of sucrose but not other sugars (Jamsran et al. 1999). Furthermore, excessive concentrations of sucrose induced stress-related genes and the production of protective agents in plants (Halford et al. 2003; Solfanelli et al. 2006), implying that sucrose may act as a signal to improve preparedness for

surviving under hush condition.

Stress-related proteins and transcripts accumulate in OWBs and IOWBs

To survive the cold season, OWBs display high tolerance against cold, freezing, and dehydration. OWBs

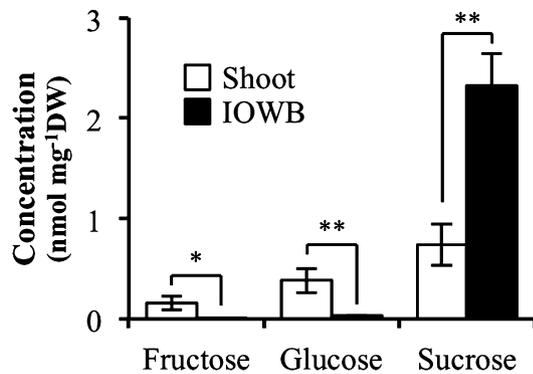


Figure 1. Sugar concentrations in shoots and IOWBs of plantlets of *G. triflora* cv. 'Ihatovo'. Values are means±SD from three independent experiments. Significance of differences was evaluated by Student's *t*-test (** $p < 0.01$, * $p < 0.05$).

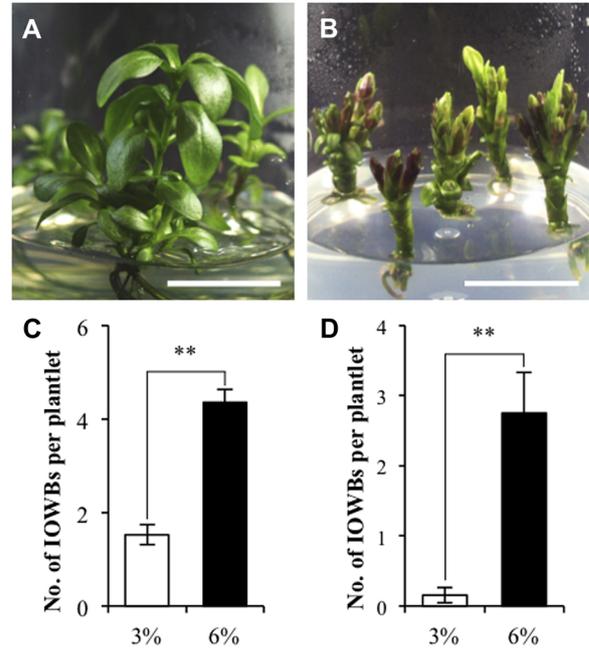


Figure 2. Photograph of in vitro plantlets (A) and IOWBs (B) of gentian cv. 'Ihatovo'. Numbers of IOWBs per plantlet (C and D). Two *G. triflora* cultivars, 'Ihatovo' (C) and 'Maciry' (D) were analyzed. White and black bars indicate standard (3%) and high sucrose conditions (6%), respectively. Values are means±SD from three independent experiments. Significance of differences was evaluated by Student's *t*-test (** $p < 0.01$). Bars indicate 2 cm.

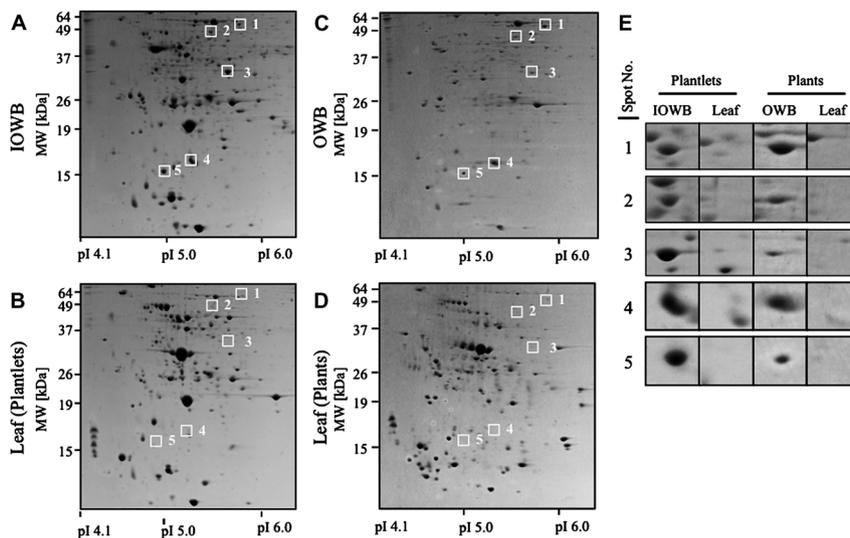


Figure 3. CBB-stained 2-D gel of proteins from *G. triflora*. Protein profiles of IOWBs (A) and leaves of plantlets (B), and protein profiles of OWBs (C) and leaves of field-grown plants (D). Leaves and OWBs of field-grown plants were harvested in May 2012 and December 2012, respectively. In plantlets 1-month-old leaves and IOWBs which had been induced by high sucrose treatment for 4 months, were harvested. Boxed protein spots were rich in IOWBs and OWBs but practically absent in leaves. (E) Enlargement of boxes from (A) to (D). Protein extracts were run on pH 4–7 IPG strips for IEF, followed by SDS-PAGE.

Table 1. List of identified proteins abundantly expressed in IOWBs.

Spot No.	Annotation ^a	pI value		Protein mass (kDa)		Matched peptide No.	Sequence coverage (%)
		Observed	Theoretical	Observed	Theoretical		
1	LEA protein	5.6	5.50	49	48.1	23	37
2	Enolase	5.4	5.29	47	48.0	12	23
3	Glyoxalase I	5.5	5.61	35	32.9	9	15
4	TPX I	5.2	5.05	17	17.4	22	24
5	Putative PR-10	4.2	4.76	15	17.1	7	19

^aLEA protein, late embryogenesis abundant protein; TPX I, thioredoxin peroxidase I; Putative PR-10, putative PR-10 type pathogenesis-related protein.

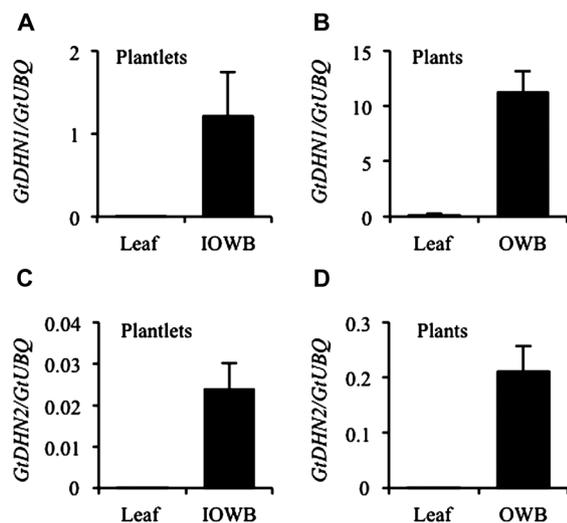


Figure 4. Expression of OWB marker genes in field-grown plants and in vitro plantlets. Expression of *GtDHNs* in in vitro plantlets (A and C) and field-grown plants (B and D). Leaves and OWBs of field-grown plants were harvested in May 2012 and October 2012, respectively. Amounts of transcripts shown are relative values normalized against transcription levels of *G. triflora Ubiquitin (GtUBQ)*. Values are means \pm SD from three independent experiments.

accumulate several stress-related enzymes and proteins such as thioredoxin peroxidase (TPX), ascorbate peroxidase (APX), and dehydrin (DHN) (Takahashi et al. 2006). We examined the protein profiles of IOWBs and leaves of gentian plantlets as well as of field-grown plants using 2D-PAGE (Figure 3A–D). Five prominent protein spots found in IOWBs and OWBs appeared absent from preparations of leaves from plantlets and plants (Figure 3E). These five spots were analyzed by LC-MS/MS and identified as highly homologous to late embryogenesis abundant (LEA) protein, enolase, glyoxalase I, TPX I, and putative PR-10 type pathogenesis-related protein, respectively (Table 1). Because homologs of these proteins are related to cold tolerance (Bahramnejad et al. 2010; Battaglia et al. 2008; Lee et al. 2002; Takahashi et al. 2006; Veena et al. 1999), IOWBs might possess a cold hardiness similar to OWBs.

Moreover, transcripts of two gentian DHNs, *GtDHN1* (accession no. AB685464) and *GtDHN2* (AB685465), were predominantly accumulated in field-grown OWBs, and overexpression of these genes enhanced dehydration stress tolerance (Imamura et al. 2013). We compared

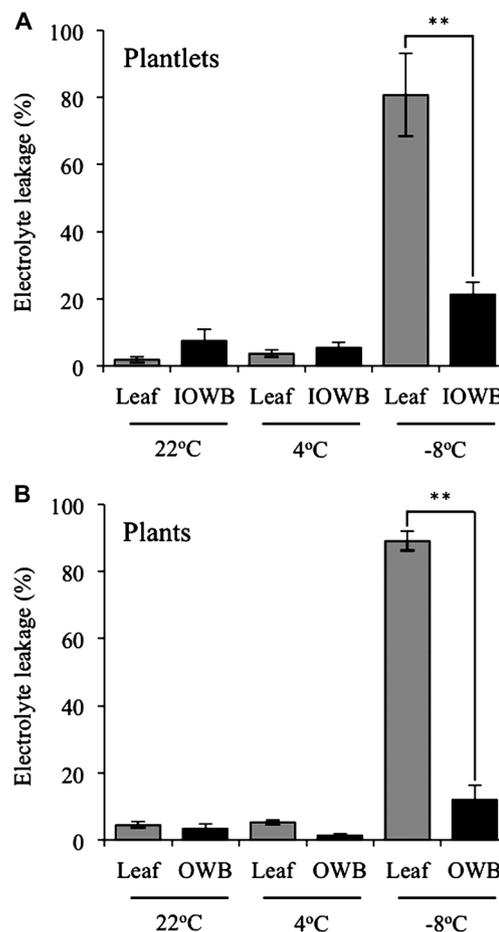


Figure 5. Freezing tolerance of plantlets and field-grown plants. (A) Electrolyte leakage induced by freezing in leaves (gray bars) and IOWBs (black bars) of gentian plantlets. (B) Electrolyte leakage induced by freezing in leaves (gray bars) and OWBs (black bars) of field-grown gentian. Leaves and OWBs of field-grown plants were harvested in May 2012 and October 2012, respectively. Electrolyte leakage was measured at 22°C, 4°C and -8°C for 3 h. Values shown are means \pm SD from three independent experiments. Significance of differences was evaluated by Student's *t*-test (** $p < 0.01$).

the accumulation of *GtDHN1* and *GtDHN2* transcripts between IOWBs and shoots of gentian plantlets by real-time RT-PCR. The expression levels were significantly higher in IOWBs than in leaves (Figure 4A and 4C). Similar results were obtained when OWBs and leaves were compared (Figure 4B and 4D). These findings supported the idea that IOWBs might have a dehydration stress tolerance similarly as OWBs.

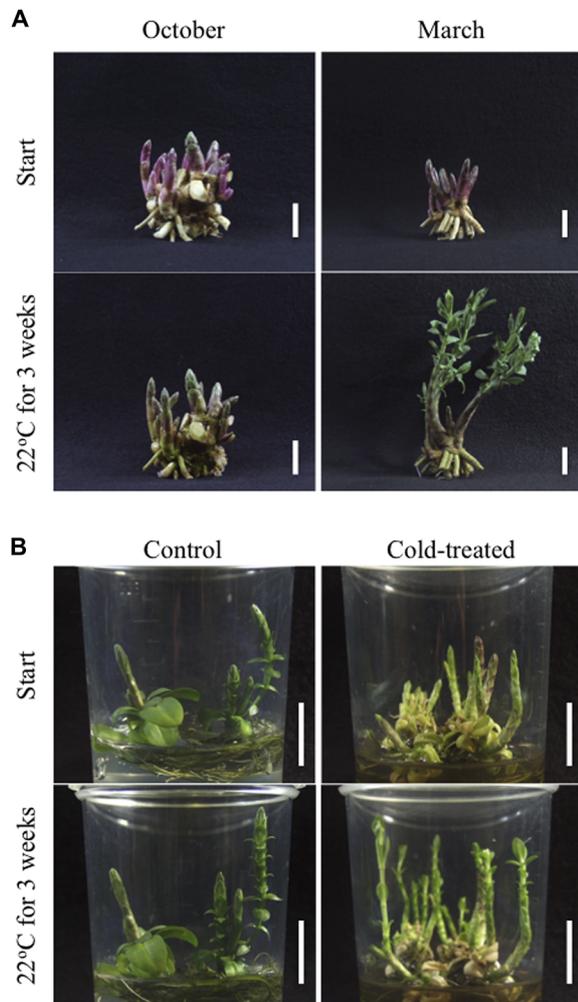


Figure 6. Evaluation of dormant states in IOWBs and OWBs. Bud break treatment was performed by incubation at 22°C for 3 weeks. (A) Photographs of gentian OWBs. Left column, OWBs harvested in October; right column, OWBs harvested in March. Upper and lower row show OWBs before and after bud break treatment, respectively. (B) Photographs of IOWBs. Cold treatment was performed by incubation at 4°C for 5 weeks (right); control material (left) remained untreated. Upper and lower row show IOWBs before and after bud break treatment, respectively. Three independent experiments were performed. Bars indicate 2 cm.

IOWB possess freezing tolerance similar to OWBs

To determine whether IOWB possess a degree of dehydration stress tolerance comparable to that of OWBs, we determined electrolyte leakage from IOWBs, OWBs plantlet leaves, and plant leaves as a measure of freezing-induced membrane injury. At 22°C and 4°C, all organs showed low electrolyte leakage (Figure 5). However, at -8°C IOWBs showed significantly less leakage compared to leaves (Figure 5A). Results from OWBs and plant leaves were similar (Figure 5B). The observed tolerance in IOWB might be due to the accumulation of *GtDHN1* and *GtDHN2* (Figure 4), and other factors. Overexpression of *GtDHN1* or *GtDHN2* improved dehydration stress tolerance (Imamura et al. 2013). Previous studies reported that overexpression of

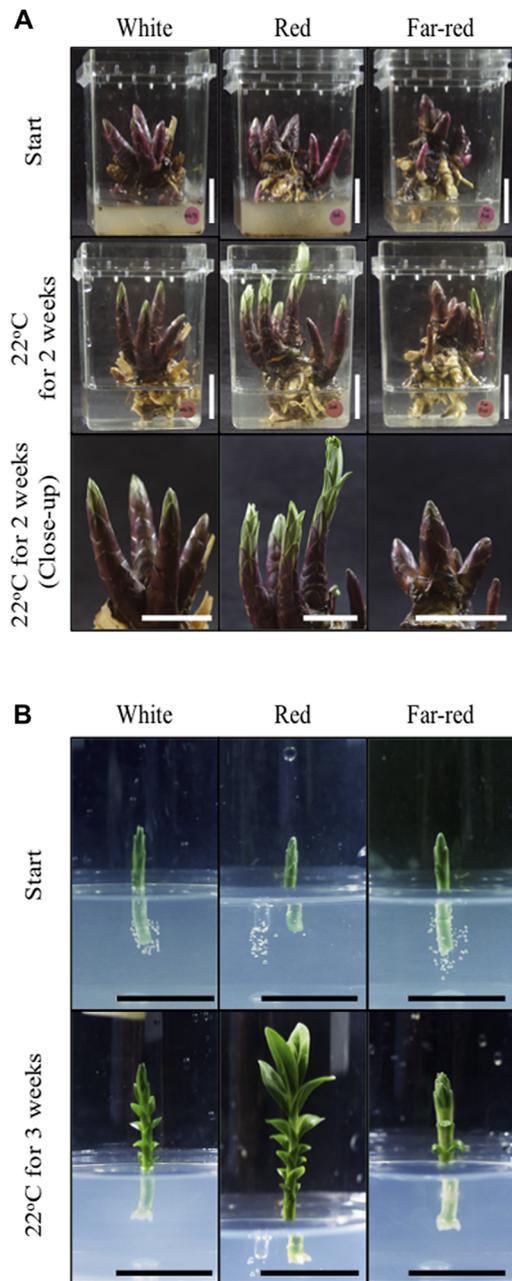


Figure 7. Effects of different wavelengths of light on bud breaking in OWBs and IOWBs from gentian. (A) Bud breaking in OWBs exposed to white (left), red (middle), and far-red light (right); the treatment was applied for 2 weeks at 22°C. Upper and middle rows show OWBs prior to and after treatment, respectively. The lower row presents close-ups of the middle row. (B) Analogous experiment using ecodormant IOWBs. Three independent experiments were performed. Bars indicate 2 cm.

DHN in other plants enhanced the resistance to freezing stress (Peng et al. 2008; Puhakainen et al. 2004). Our results indicated that IOWBs resembled OWBs in terms of freezing tolerance.

Long-term cold treatment induces endodormancy release in IOWBs

First, we characterized the dormant state of OWBs (Figure 6A). After incubation at 22°C for 3 weeks, OWBs

in tests conducted in March sprouted, whereas OWBs in tests performed in October did not and remained dormant. This result suggested that OWBs in October and March were in endodormancy and ecodormancy, respectively. Next, to reveal their dormant states, IOWBs were cold-treated (4°C) for 5 weeks and then were incubated at 22°C for 3 weeks to induce bud break. Cold-treated IOWBs sprouted whereas non-treated control IOWBs remained dormant (Figure 6B), suggesting that control IOWBs were in endodormancy whereas cold-treated IOWBs were in ecodormancy. These results indicated that IOWBs had similar dormancy mechanisms as OWBs.

Effect of different wavelengths of light on bud breaking

Previous reports showed that bud dormancy release depended on light quality similarly as seed germination does (Chao et al. 2007). To study the possible involvement of light quality on bud break of gentian, ecodormant OWBs were exposed to red, far-red, and white light (40 μmol m⁻² s⁻¹). Two weeks after exposure, a larger proportion of bud breaking was observed under red than under white light, while under far-red light, the buds did not sprout (Figure 7A). Next, we assessed the effect of light quality on bud breaking in ecodormant IOWBs. Three weeks after red-light exposure, bud break, whereas far-red light had no effect (Figure 7B). Thus, IOWBs and OWBs responded similarly to red and far-red light. Our results suggested that bud breaking in gentian is controlled by phytochrome-mediated switch, similarly as seed germination.

Conclusions

In this study, we established method of IOWB induction in gentian plantlets by culturing on 6% sucrose medium. Our method works reliably even in cultivars that rarely produce IOWBs under standard conditions. Since IOWBs are dormant and grow much slower than plantlets, they are the preferred material for long-term maintenance. IOWBs resemble OWBs with respect to *DHN* expression, freezing tolerance, endodormancy release, and response to red or far-red light (Figures 4–7). In this study, we demonstrated not only the induction of dormant buds in vitro, but also the physiological similarity between IOWBs and OWBs. Since, IOWBs can be cultured easily under a variety of conditions, they represent a convenient material for studying the regulation of phenomena occurring in OWBs such as dormancy, transition of dormant states, and bud breaking.

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