Change of supercooling capability in solutions containing different kinds of ice nucleators by flavonol glycosides from deep supercooling xylem parenchyma cells in trees

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ABSTRACT Deep supercooling xylem parenchyma cells (XPCs) in katsura tree contain flavonol glycosides with high supercooling-facilitating capability in solutions containing the ice nucleation bacterium (INB) *Erwinia ananas,* which is thought to have an important role in deep supercooling of XPCs. The present study, in order to further clarify the roles of these flavonol glycosides in deep supercooling of XPCs, the effects of these supercooling-facilitating (anti-ice nucleating) flavonol glycosides, kaempferol 3-*O*-β-D-glucopyranoside (K3Glc), kaempferol 7-*O*-β-D-glucopyranoside (K7Glc) and Quercetin 3-*O*-β-D-glucopyranoside (Q3Glc), in buffered Milli-Q water (BMQW) containing different kinds of ice nucleators, including INB *Xanthomonas campestris*, silver iodide and phloroglucinol, were examined by a droplet freezing assay. The results showed that all of the flavonol glycosides promoted supercooling in all solutions containing different kinds of ice nucleators, although the magnitudes of supercooling

capability of each flavonol glycoside changed in solutions containing different kinds of ice nucleators. On the other hand, these flavonol glycosides exhibited complicated nucleating reactions in BMQW, which did not contain identified ice nucleators but contained only unidentified airborne impurities. Q3Glc exhibited both supercooling-facilitating and ice nucleating capabilities depending on the concentrations in such water. Both K3Glc and K7Glc exhibited only ice nucleation capability in such water. It was also shown by an emulsion freezing assay in BMQW that K3Glc and Q3Glc had no effect on homogeneous ice nucleation temperature, whereas K7Glc increased ice nucleation temperature. The results indicated that each flavonol glycoside affected ice nucleation by very complicated and varied reactions. More studies are necessary to determine the exact roles of these flavonol glycosides in deep supercooling of XPCs in which unidentified heterogeneous ice nucleators may exist.

Key words: Supercooling of water, xylem parenchyma cells (XPCs), homogenous and/or heterogeneous ice nucleation, flavonol glycosides

Introduction

Xylem parenchyma cells (XPCs) of trees adapt to subfreezing temperatures by deep supercooling, which is a unique freezing adaptation mechanism for maintaining a liquid state of cellular water without dehydration at very low sub-zero temperatures for a long period [6,17,5]. Such deep supercooling XPCs have developed mechanisms to change the temperature limit of supercooling to below environmental temperatures in order to avoid lethal intracellular freezing by breakdown of supercooling. The temperature limit of supercooling in XPCs of trees gradually decreases in parallel with latitudinal environmental temperature reduction in ranges from -10 ˚C in trees growing in tropical areas to around -60 ˚C in trees growing in cold areas during winter [6,17]. The temperature limit of supercooling in XPCs is also changed by seasonal environmental temperature fluctuations. In boreal trees, the temperature limit of supercooling changes from -20 ˚C during summer to around -60 ˚C during winter [6]. In katsura trees growing in a temperate area, the temperature limit of supercooling in XPCs changes from -20 ˚C during summer to -40 ˚C during winter [11].

The supercooling mechanisms in XPCs were previously referred to the physical state of water [1], based on results of physical experiments showing that a small isolated water droplet without containing heterogeneous ice nucleators supercools to -38 ˚C,

which corresponds to the homogeneous ice nucleation temperature of water [4,18]. However, recent studies have shown that it may be difficult to explain the deep supercooling phenomenon in XPCs, especially the mechanism by which the temperature limit of supercooling in XPCs fluctuates, only by a physical water isolation theory [5].

Recent studies have indicated important roles of intracellular substances in the deep supercooling phenomenon of XPCs. It has been shown that artificial release of intracellular substances from XPCs significantly reduced supercooling capability in beech XPCs [13]. It has also been reported that the fluctuations of supercooling capability in XPCs are associated with changes in expression of many genes in larch [20], changes in accumulation of many proteins in several trees [5] and changes in accumulation of soluble carbohydrates in birch [9], although the direct roles of these physiological changes in deep supercooling of XPCs remain to be clarified.

To investigate roles of intracellular substances in deep supercooling of XPCs, an approach to search for supercooling-facilitating (anti-ice nucleation) substances in XPCs has been started. Crude extracts from xylem tissues that include deep supercooling XPCs in several hardwood species including katsura tree exhibited supercooling-facilitating capability in solutions containing the ice nucleation bacterium (INB) *Erwinia ananas* [12]. Furthermore, novel types of supercooling-facilitating

substances were identified in crude xylem extracts from katsura tree [11]. These supercooling-facilitating substances included four kinds of flavonol glycosides, kaempferol 3-*O*-β-D-glucopyranoside (K3Glc), kaempferol 7-*O*-β-D-glucopyranoside (K7Glc), 8-methoxykaempferol 3-*O*-β-D-glucopyranoside (8mK3Glc) and quercetin 3-*O*-β-D-glucopyranoside (Q3Glc), which exhibited high capability to promote supercooling in solutions containing the INB *E. ananas* and suggested an important role of these flavonol glycosides in deep supercooling of XPCs [11].

It has been pointed out that in a biological system, internal water is inevitably in contact with other surface, so that nucleation is justifiably assumed to be heterogeneous [22]. Activity of heterogeneous ice nucleation in supercooling XPCs of shagbark hickory has been reported [7]. Furthermore, the temperature limit of supercooling in XPCs is much higher than the homogeneous ice nucleation temperature under warm environmental temperatures. Therefore, it is thought that fluctuation of the temperature limit of supercooling in XPCs may be related to interaction between supercooling-facilitating substances and heterogeneous ice nucleators in XPCs, although the nature of ice nucleators in XPCs is unclear.

It was found in previous studies that in already-reported supercooling-facilitating substances, the supercooling capability changes depending on the kinds of ice nucleator

in solutions [12,25,26]. Therefore in order to elucidate the effect of flavonol glycosides on control of supercooling capability in XPCs, it is necessary to determine the effects of these flavonol glycosides on solutions containing different kinds of ice nucleators. In this study, we examined the supercooling effects of flavonol glycosides, including K3Glc, K7Glc and Q3Glc that exist in deep supercooling XPCs in katsura tree on solutions containing different kinds of ice nucleators. The results of the present study showed very complex interactions of these flavonol glycosides in solutions containing different kinds of ice nucleators, suggesting complicated mechanisms to control deep supercooling in XPCs by these flavonol glycosides.

Materials and methods

Preparation of supercooling-facilitating flavonol glycosides

Synthetic flavonol glycosides, K3Glc, K7Glc and Q3Glc, were purchased from Extrasynthèse (Genay, France), and they were used throughout all examinations without further purification. We previously confirmed that these synthetic flavonol glycosides had the same supercooling capability as that of flavonol glycosides purified from xylem

of katsura tree [11].

Preparation of ice nucleators

UV-sterilized and lyophilized INB *Erwinia ananas* and lyophilized cell debris of INB *Xanthomonas campestris* were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and they were used without further treatment. Each lyophilized INB was added at a concentration of 2 mg/mL to 50 mM phosphate buffer (pH 7.0).

Twenty mM solution of potassium iodide (Nacalai Tesque, Japan) was mixed with an equal volume of 20 mM solution of silver nitrate (WAKO Pure Chemical Industries, Osaka, Japan). The resultant solution containing colloidally dispersed silver iodide (AgI) particles was diluted twentyfold in 50 mM phosphate buffer (pH 7.0).

Phloroglucinol anhydrous (Tokyo Chemical Industry, Co., Ltd.) was added to water at a concentration of 396 mM and heated at 65 ˚C for 10 min and then sonicated until dissolved. This solution was immediately diluted in 50 mM phosphate buffer (pH 7.0) to 119 mM.

Measurement of ice nucleation temperatures by a droplet freezing assay

Ice nucleation temperatures of solutions were analyzed by a droplet freezing assay described by Vali [21] with some modifications. The solutions consisted of diluted phosphate buffer solution (50 mM potassium phosphate, pH 7.0) in Milli-Q water, buffered Milli-Q water (BMQW). The use of such buffer solutions throughout all examinations in this study made flavonol glycoside soluble and the freezing behavior of such buffer solutions was the same with Milli-Q water alone [12].

One kind of ice nucleator and one kind of flavonol glycoside, depending on the purpose, were added to BMQW. When identified ice nucleators were added to BMQW, their final concentrations were 2 mg/mL in *E. ananas*, 1 mg/mL in *X. campestris*, 0.5 mM in silver iodide and 119 mM in phloroglucinol. Also, one kind of flavonol glycoside was added to BMQW in which no identified ice nucleators were added but contained only airborne impurities (unidentified ice nucleators) that were unintentionally included during the process of the experiment.

Two μL of solution in a droplet was placed on the surface of a copper plate coated with mineral oil (Nacalai Tesque Ltd., Kyoto, Japan). The copper plate with numerous droplets was set in an alcohol bath (F26, Julabo Labortechnik GmbH, Seelback, Germany) maintained at 0 °C and immediately cooled at a rate of 0.2 °C/min to -30 °C.

Freezing (nucleation) of droplets was judged by the naked eye. The percentage of cumulative number of frozen droplets with 0.5 °C temperature decrements was plotted as the cumulative ice nucleation spectrum. In each treatment, 100 to 120 droplets in total from 4 to 5 examinations were counted [21]. The temperature required for freezing 50% of the droplets was indicated as INT_{50}, ice nucleation temperature for 50% droplets. The supercooling capability is expressed by the difference between INT_{50} for samples with addition of flavonol glycosides and that for samples without addition of flavonol glycosides. The freezing curves obtained by the droplet freezing assay using 100 to 120 droplets and resultant values of INT_{50} were repeatedly confirmed by different replications and the most typical and representative data are shown.

Measurement of ice nucleation temperatures by an emulsion freezing assay

Emulsions of BMQW were prepared in silicone oil (Element14*, Momentive performance materials, Tokyo, Japan) containing 5% (w/w) of SPAN 65 (Wako Pure Chemical, Osaka, Japan) as an emulsifier. BMQW contained each flavonol glycoside at a concentration of 0.02% (w/v). Such an aqueous solution in silicone oil (BMQW / silicone oil = 1 / 2) was emulsified by sonication with an ultrasonic cell disruptor

(XL2000, Misonix Inc., NY, USA). Micro-droplets of less than 10 μm in diameter were prepared in emulsions with confirmation of droplet size by light microscopic observation (BH-2, Olympus, Tokyo, Japan). Forty μL of an emulsified sample was put into a micro test tube, and a copper-constantan thermocouple (AUG #36) was taped on the outside of each micro test tube. To determine the temperature of freezing in each sample by differential thermal analysis (DTA), the differences between thermal responses of emulsified samples and silicone oil were recorded during cooling from 4 °C to –50 °C at a rate of 0.2 °C/min. Temperatures of exothermal peaks of micro-droplets are presented as means ± SE from five measurements.

Results

Supercooling capability of flavonol glycosides in solutions containing different kinds of ice nucleators by a droplet freezing assay

Our previous study showed that flavonol glycosides, K3Glc, 8mK3Glc, K7Glc and Q3Glc, that found in deep supercooling XPCs of katsura tree promoted supercooling in solutions containing the INB *E. ananas* [11]. In the present study, we examined

supercooling capability of K3Glc, K7Glc and Q3Glc in solutions containing different kinds of ice nucleation substances including the INB *X. campestris*, silver iodide and phloroglucinol, in addition to the INB *E. ananas* for comparison.

Flavonol glycosides of a concentration at 0.05% (w/v) exhibited supercooling capability in all solutions containing different kinds of ice nucleators, INB *E. ananas*, INB *X. campestris*, silver iodide and phloroglucinol (Fig. 1a-d). However, the supercooling capability of each flavonol glycoside changed markedly depending on the kind of ice nucleator in the solution. As was reported previously [11], K7Glc had the highest supercooling capability of 7.3 ˚C in solutions containing INB *E. ananas*, while K3Glc and Q3Glc showed supercooling capabilities of 4.8 ˚C and 3.3 ˚C, respectively (Fig. 1a). In solutions containing INB *X. campestris* (Fig. 1b), K7Glc also exhibited the highest supercooling capability of 3.0 ˚C, although the supercooling capability became less than that in solutions containing INB *E. ananas*. Q3Glc and K3Glc showed supercooling capabilities of 1.4 ˚C and 1.0 ˚C, respectively, in solution containing INB *X. campestris* (Fig. 1b). In solutions containing silver iodide (Fig. 1c), K3Glc showed the highest supercooling capability of 4.6 ˚C, while Q3Glc and K7Glc showed supercooling capabilities of 4.2 ˚C and 2.0 ˚C, respectively. In solution containing phloroglucinol (Fig. 1d), Q3Glc showed the highest supercooling capability of 3.2 ˚C,

while K7Glc and K3Glc showed supercooling capabilities of 1.0 ˚C and 0.5 ˚C, respectively.

Flavonol glycosides of a reduced concentration at 0.01% (w/v) resulted in reduced magnitude of supercooling capability compared to that at a concentration at 0.05% (w/v) in solutions containing different kinds of ice nucleators (Fig. 1e-h). In solutions containing INB *E. ananas* (Fig. 1e), K7Glc, Q3Glc and K3Glc showed supercooling capabilities of 5.7 ˚C, 2.0 ˚C and 1.7 ˚C, respectively. In solution containing INB *X. campestris* (Fig. 1f), K7Glc, K3Glc and Q3Glc showed supercooling capabilities of 1.5 ˚C, 0.2 ˚C and 0 ˚C, respectively. In solutions containing silver iodide (Fig. 1g), Q3Glc, K3Glc and K7Glc showed supercooling capability of 3.2 ˚C, 1.8 ˚C and 0.2 ˚C, respectively. In solution containing phloroglucinol (Fig. 1h), Q3Glc, K3Glc and K7Glc showed supercooling capabilities of 1.6 ˚C, 0.4 ˚C and 0 ˚C, respectively. Further reduction of the concentration of these flavonol glycosides to 0.001% (w/v) resulted in almost disappearance of supercooling capability in all solutions containing different kinds of ice nucleators (results not shown).

Supercooling capability of flavonol glycosides in BMQW without containing identified ice nucleators by a droplet freezing assay

The effects of flavonol glycosides on supercooling of BMQW without addition of identified ice nucleators were examined. This examination was planned to test the effects of supercooling-facilitating flavonol glycosides on unidentified airborne ice nucleators (impurities). Such water was frozen at -22.7 ± 0.6 ˚C (INT_{50}, mean ± SD, n = 3), suggesting freezing by heterogeneous nucleation by airborne ice nucleators (Fig. 2). Flavonol glycosides exhibited very complex reaction on freezing of such BMQW.

In BMQW containing unidentified airborne ice nucleators, K3Glc and K7Glc became ice nucleators but not supercooling-facilitating substances. K3Glc promoted ice nucleation in BMQW (Fig. 2a). While BMQW alone supercooled to -22.7 ˚C, addition of K3Glc in BMQW at a concentration of 0.05% produced freezing at –16.9 ˚C showing ice nucleation activity of 5.8 ˚C. Ice nucleation activity by addition of K3Glc was gradually decreased by reducing the concentration of K3Glc to 0.1 ˚C at concentration of 0.001% (Fig. 2a). Similar to but more distinctly than K3Glc, K7Glc became a strong ice nucleator (Fig. 2b). While BMQW alone supercooled to -22.1 ˚C, addition of K7Glc in BMQW at a concentration of 0.05% produced freezing at -8.3 ˚C showing ice nucleation activity of 13.8 ˚C. The ice nucleation activity was also gradually decreased by reducing the concentration of K7Glc to 3.5 ˚C at concentration of 0.001% (Fig. 2b).

The ice nucleation activity by K3Glc was not linear relation depending on the concentration (Fig. 3a), but the activity by K7Glc seems to be linear relation depending on the concentration (Fig. 3b).

Q3Glc showed complex reaction on freezing of BMQW (Fig. 2c). Q3Glc exhibited both supercooling-facilitating and ice nucleation activities in BMQW depending on the concentration. Q3Glc showed supercooling capability of 1.4 ˚C at concentration of 0.05% but showed ice nucleation activity of 3.5 ˚C at a concentration of 0.01%. However, by further reduction of the concentration, Q3Glc showed ice nucleation activity of 2.9 ˚C at a concentration of 0.005% but again showed supercooling capability of 1.4 ˚C at a concentration of 0.001% (Fig. 3c). We repeated measurements of the supercooling capability of flavonol glycosides to BMQW several times using different butches of Q3Glc solutions and obtained similar results (Fig. 3).

Effects of flavonol glycosides on homogenous ice nucleation by an emulsion freezing assay

An emulsion freezing assay was used to test the effects of flavonol glycosides on homogenous ice nucleation (Fig. 4). In this assay, emulsified micro-droplets of BMQW

alone showed ice nucleation at around -37.9 ± 0.1 ˚C (mean ± SE, n = 5), corresponding to the homogeneous ice nucleation temperature of water (Fig. 4a). Emulsified micro-droplets of BMQW containing K3Glc (Fig. 4b) or Q3Glc (Fig. 4d) at a concentration of 0.02% (w/v) did not affect the homogenous ice nucleation temperature, showing peaks of the freezing exotherm at around -37.6 ± 0.1 ˚C and -37.4 ± 0.2 ˚C, respectively. On the other hand, emulsified micro-droplets of BMQW containing K7Glc at a concentration of 0.02% increased the ice nucleation temperature, showing a broad peak of the freezing exotherm at around -32.9 ± 0.2 ˚C, although a second sharp peak was also seen at around -37.7 ± 0.1 ˚C (Fig. 4c).

Discussion

Our previous study showed that deep supercooling XPCs in katsura tree contained supercooling-facilitating (anti-ice nucleating) flavonol glycosides, such as K3Glc, K7Glc and Q3Glc [11]. In our previous study, however, the supercooling-facilitating activity of flavonol glycosides was confirmed in a solution containing the INB *E. ananas* alone [11]. In many previous studies in which supercooling-facilitating substances were found, the supercooling activities were also confirmed by using

solutions containing one kind of ice nucleators: the supercooling capability of 55-kDa proteins from *Acinetobacter calcoaceticus* was confirmed in a solution containing the INB *E. uredovora* KUIN-3 alone [15]; the supercooling capabilities of several kinds of terpenoids, including hinokitiol, hinokitin, α-pinene, α-terpinene and limonene, were confirmed in solutions containing the INB *Pseudomonas fluorescens* KUIN-1 alone [14]; the supercooling capabilities of several kinds of phenylpropanoids, including eugenol, α-methoxyphenol, 2-allylphenol and 4-allylanisole, were confirmed in solutions containing the INB *E. uredovora* KUIN-3 alone [16]; the supercooling capabilities of seed extracts were confirmed in solutions containing silver iodide alone [2]; and the supercooling capabilities of 12 kinds of flavonol glycosides, including kaempferol 3-*O*-ß-D-rutinoside, kaempferol 7-*O*-ß-D-galactopyranoside, quercetin 5-*O*-ß-D-glucopyranoside, quercetin 7-*O*-ß-D-glucopyranoside, quercetin 3'-*O*-ß-D-glucopyranoside, quercetin 4'-*O*-ß-D-glucopyranoside, quercetin 3-*O*-ß-D-galactopyranoside, quercetin 7-*O*-ß-D-galactopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside, quercetin 3-*O*-ß-D-rutinoside, myricetin 3-*O*-ß-D-glucopyranoside and myricetin 3-*O*-α-L-rhamnopyranoside, were confirmed in solutions containing the INB *E. ananas* alone [10].

The present study showed that the flavonol glycosides K3Glc, K7Glc and Q3Glc

exhibited supercooling-facilitating capability in all solutions containing different kinds of ice nucleators including not only the INB *E. ananas* as shown in a previous study [11] but also the INB *X. campestris*, silver iodide and phloroglucinol, although the magnitude of supercooling capability of each flavonol glycoside was different depending on the kind of ice nucleator in the solution (Fig. 1). Similar results showing multiple effects of supercooling-facilitating substances on different kinds of ice nucleators have also been obtained in previous studies. Supercooling capability of antifreeze proteins (AFP) from *Dendroides canadensis* was confirmed not only in solutions containing protein-like ice nucleators but also in solutions containing the INB *P. syringae*, showing supercooling capabilities of 1.7 ˚C and 1.2 ˚C (Hereafter supercooling capability in the literature is also presented by the difference of INT_{50} with the control.), respectively, although AFP concentration was not shown [3]. Supercooling capability of antifreeze glycoprotein (AFGP) from the Antarctic notothenioid *Dissostichus mawsoni* was confirmed in a solution containing INB *Erwinia herbicola*, showing supercooling of 1.0 ˚C at 1 mg/mL [19], and in a solution containing *Hemideina maori* hemolymph as an ice nucleator, showing supercooling of 2.5 ˚C at 10 mg/mL [23]. Polypeptides of 130 kDa from *Bacillus thuringiensis* showed a wide range of supercooling capabilities from 0.1 ˚C to 4.2 ˚C depending on the kinds of ice

nucleator in the solution, such as the INB *Pantoea ananas* KUIN-3, INB *X. translucens* IFO 13558, INB *Pseudomonas fluorescens* KUIN-1, silver iodide, metaldehyde, fluoren-9-one and phenazine [26]. In our previous study, it was also found that crude xylem extracts from katsura tree at a concentration of 100 mosmol/kg showed wide ranges of supercooling capabilities from 0.3 ˚C to 1.9 ˚C depending on the kind of ice nucleator in the solution, such as the INB *E. ananas,* INB *P. syringae,* INB *X. campestris*, and silver iodide [12]. Furthermore, it has also been reported that the numbers of ice nucleation sites during devitrification of 55% ethylene glycol solutions containing different kinds of ice nucleators, such as phloroglucinol, 3-aminophenol, phenazine, metaldehyde, phthalic anhydride, acetoacetanilide and 2-nitrodiphenylamine, are widely varied in the presence of 0.1% (w/w) polyvinyl alcohol (PVA), suggesting PVA has different anti-ice nucleation (supercooling-facilitating) capabilities for different kinds of ice nucleators [25]. That study also showed that 0.1% (w/w) polyglycerol (PGL) is an effective anti-ice nucleator in a solution containing INB *P. syringae* 31A but is not effective in solutions containing other kinds of ice nucleators [25].

The present study also showed that supercooling capability of flavonol glycosides in solutions containing identified ice nucleators increased with higher concentrations of flavonol glycosides, although it was not linear relation depending on the concentration

(Fig. 1). The effect of concentration of supercooling-facilitating substances on supercooling in solutions containing identified ice nucleators has been examined in only a few previous studies. In all of those previous studies examining the effects of supercooling-facilitating PGL at concentrations from 0.001 to 1.0% (w/w) in solutions containing INB *P. syringae* 31A [25], supercooling-facilitating PVA at concentrations from 0.1 to 1.0% (w/w) in solutions containing the INB *P. syringae* [8], and supercooling-facilitating flavonol glycosides including K3Glc, 8mK3Glc, K7Glc and Q3Glc at concentrations from 0.01 mg/mL to 1 mg/mL in solutions containing the INB *E. ananas* [11], an increase in supercooling capability at higher concentrations of the supercooling-facilitating substances was found as in the present study (Fig. 1).

On the other hand, the present study showed very complex responses of flavonol glycosides, Q3Glc, K7Glc and K3Glc, to ice nucleation of BMQW in which only airborne impurities were unintentionally included (Fig. 2). In solutions containing only unidentified ice nucleators without addition of identified ice nucleators, previous studies have shown supercooling-facilitating capability of 4.9 ˚C in tap water by addition of 1.0% (w/w) AFGP from the Antarctic notothenioid *D. mawsoni* [8], supercooling-facilitating capability of 1.5 ˚C in tap water by addition of 0.5% (w/w) AFP III from the fish *Macrozoarces americanus* [8], supercooling-facilitating capability

of 4.6 ˚C in tap water by addition of 1.0% (w/w) PVA [8], supercooling-facilitating capability around 1 ˚C in natural lake water by addition of 0.1% (w/w) PVA [25], supercooling-facilitating capability around 2 ˚C in a large volume (22 mL) of laboratory water by addition of 1% (w/w) PGL, suggesting contamination by an INB in a large volume of laboratory water [25], and supercooling-facilitating capability of 1.7 ˚C in buffered Milli-Q water by addition of 100 mosmol/kg crude xylem extracts from katsura tree [12]. Conversely, it has also been reported that 130-kDa polysaccharides from *B. thuringiensis* showed no supercooling-facilitating capability in distilled water, while those polysaccharides enhanced supercooling in solutions containing different kinds of identified ice nucleators [26]. Furthermore, Wowk and Fahy [25] showed the absence of supercooling-facilitating capability in a small amount of natural lake water by addition of 0.1% (w/w) PGL which has specific supercooling-facilitating capability only in a solution containing an INB, suggesting no contamination by an INB in such a small amount of lake water.

Among the flavonol glycosides examined in this study, K3Glc and K7Glc were very effective supercooling-facilitating substances in all solutions containing a wide variety of identified ice nucleators (Fig. 1), whereas, surprisingly, they were strong ice nucleators in BMQW containing only unidentified airborne ice nucleators (Figs. 2a and

b, Figs. 3a and b). Results of emulsion freezing assays also showed that K3Glc did not affect homogeneous ice nucleation temperature, while K7Glc strongly enhanced ice nucleation (Fig. 4). These results suggested that K3Glc becomes an ice nucleator by interaction with airborne ice nucleators but that K3Glc alone is not an ice nucleator, whereas K7Glc becomes an ice nucleator by itself, although K7Glc was a very effective supercooling-facilitating (anti-ice nucleation) substances in solutions containing identified ice nucleators (Fig. 1). Such an example of the same supercooling-facilitating substance having both supercooling-facilitating and ice nucleation-enhancing activities due to the difference in ice nucleators is rare. Only a preliminary study by Holt [8] has shown that AFGP from *D. mawsoni* or AFP III from *M. americanus* promoted supercooling in tap water, whereas they enhanced ice nucleation in solution containing the INB *P. syringae*.

Q3Glc showed more complex ice nucleation activities in BMQW containing only unidentified airborne ice nucleators (Fig. 2c). Q3Glc promoted supercooling at concentrations of 0.05% and 0.001% (w/v) but enhanced ice nucleation at concentrations of 0.01% and 0.005% (w/v) (Fig. 3c). Such a concentration-dependent difference in ice nucleation activities has also been previously reported. A preliminary study by Holt [8] showed that AFP III exhibited both supercooling and ice nucleation

activities depending on concentrations in solutions containing *P. syringae* or in tap water. Type I AFP from *Pleuronectes americanus* gradually enhances supercooling capability in distilled water when the concentration is increased from 1 to 8 mg/mL but oppositely gradually reduces the supercooling capability at higher concentrations [24]. It was suggested that ice nucleation activity at higher concentrations of Type I AFP may be produced by aggregation of AFP, which may act as ice nucleators [24]. However, this is not the case in Q3Glc, because Q3Glc promoted supercooling at the highest concentration (0.05%) and lowest concentration (0.001%) but enhanced ice nucleation capability at intermediate concentrations (0.01 and 0.005%) (Figs. 2c and 3c). Q3Glc did not affect homogeneous ice nucleation temperature (Fig. 4). Thus, it is likely that there are very complicated interactions of Q3Glc with unidentified airborne ice nucleators more than simple aggregation of Q3Glc.

The present study clarified complicated reactions of flavonol glycosides, K3Glc, K7Glc and Q3Glc, which exist in deep supercooling XPCs, in solutions containing different kinds of ice nucleators. The fluctuation of supercooling capability in XPCs may be caused by interaction between supercooling-facilitating substances in XPCs and ice nucleators in XPCs, although the nature of ice nucleators is currently unknown. Thus, more studies on ice nucleation mechanisms by interactions between

supercooling-facilitating substances and ice nucleators in XPCs are necessary in order to understand the roles of supercooling-facilitating flavonol glycosides in deep supercooling of XPCs.

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Figure Legends

Fig. 1. Freezing curves of droplets in solutions containing K3Glc (white triangles),

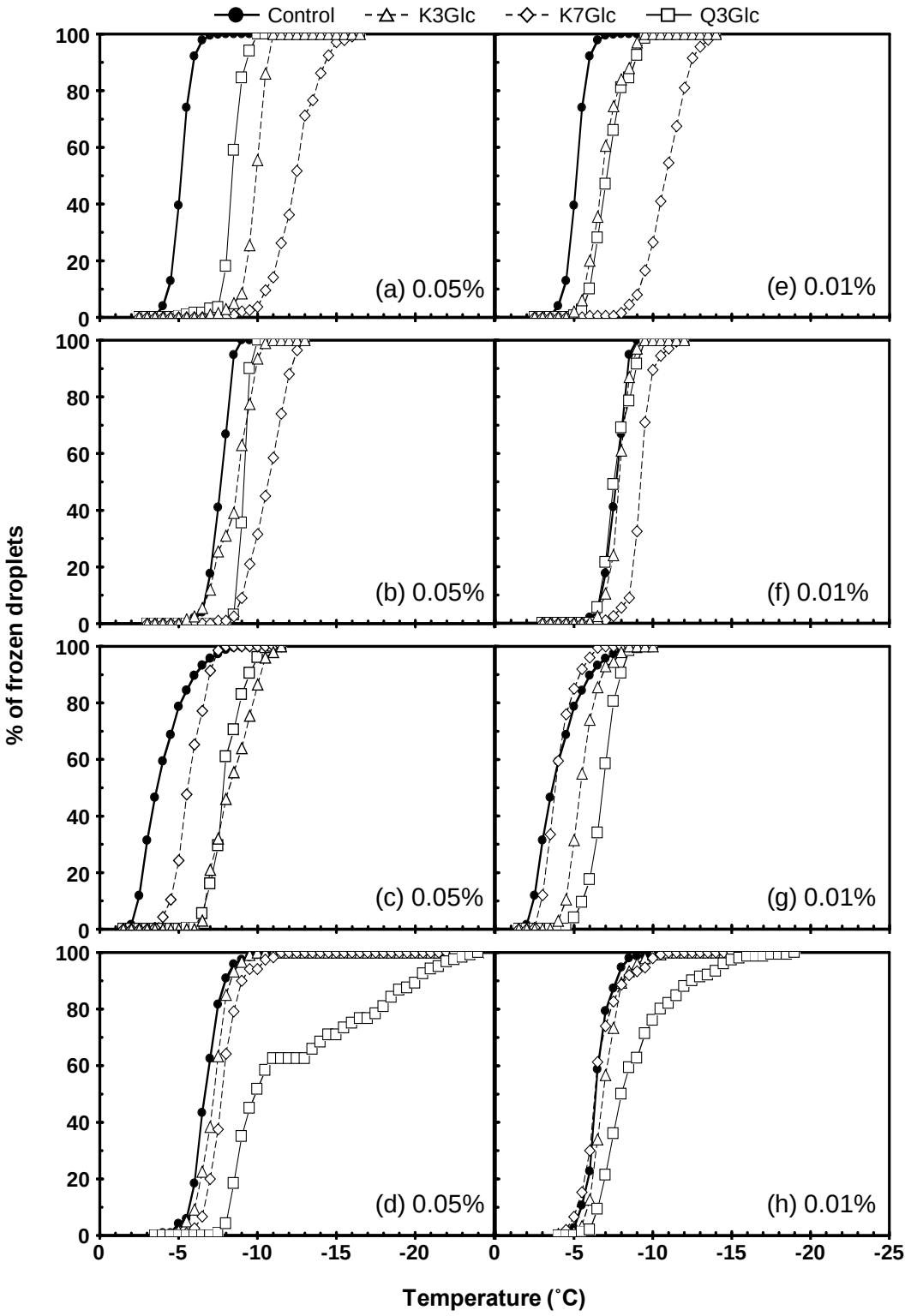
K7Glc (white diamonds) or Q3Glc (white squares) at concentrations of 0.05% (w/v) (a-d) and 0.01% (w/v) (e-h). The solutions also contained the INB *E. ananas* (a, e), INB *X. campestris* (b, f), silver iodide (c, g) or phloroglucinol (d, h). Freezing curves of control without addition of flavonol glycosides (dark circles) are also shown. For details, see Materials and methods.

Fig. 2. Freezing curves of droplets in solutions containing K3Glc (a), K7Glc (b) and Q3Glc (c) at concentrations of 0 (dark circles), 0.001 (white triangles), 0.005 (white squares), 0.01 (gray triangles) and 0.05% (w/v) (gray squares) in BMQW that contained only unintentionally included unidentified airborne ice nucleators. For details, see Materials and methods.

Fig. 3. INT_{50} of droplets in solutions containing different concentrations of K3Glc (a), K7Glc (b) and Q3Glc (c) in BMQW that contained only airborne ice nucleators. Bars = mean ± SD. n = 5.

Fig. 4. DTA profiles of emulsion freezing assay. (a) Emulsified micro-droplets of BMQW without flavonol glycosides (control). Emulsified micro-droplets of BMQW

containing 0.02% (w/v) K3Glc (b), 0.02% (w/v) K7Glc (c), and 0.02% (w/v) Q3Glc (d). For details, see Materials and methods.



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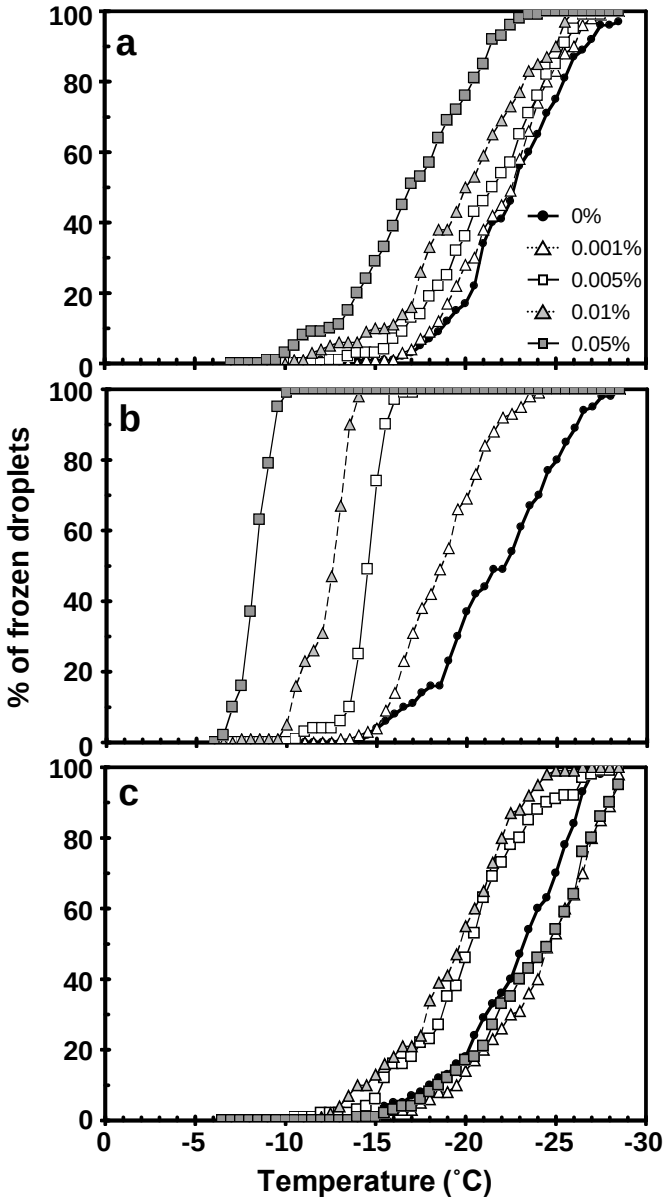

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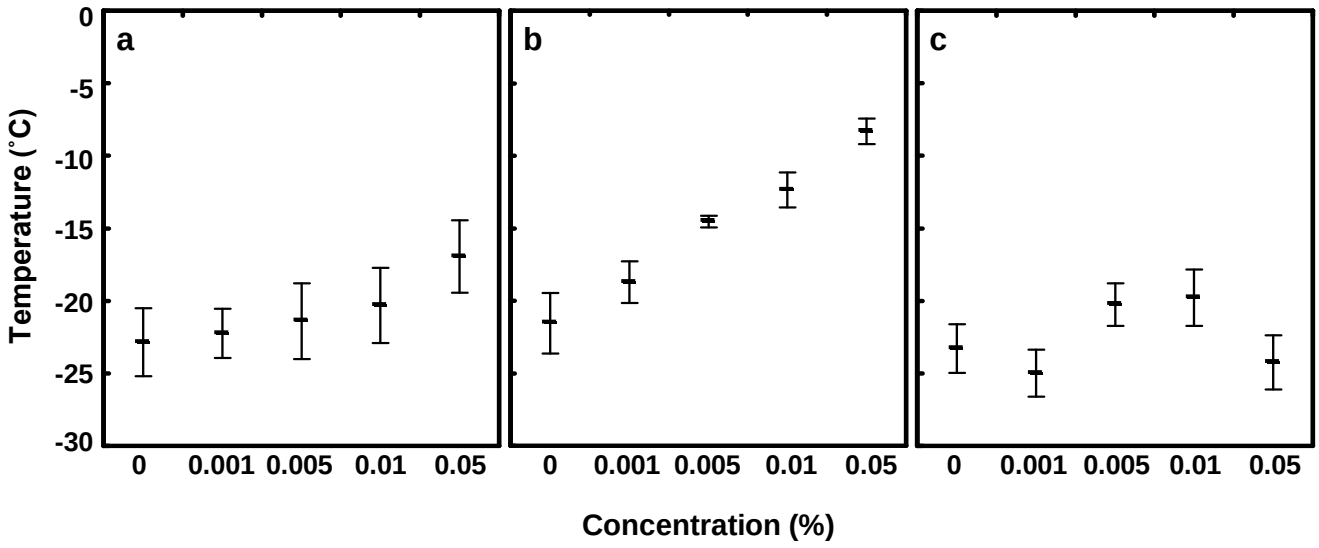

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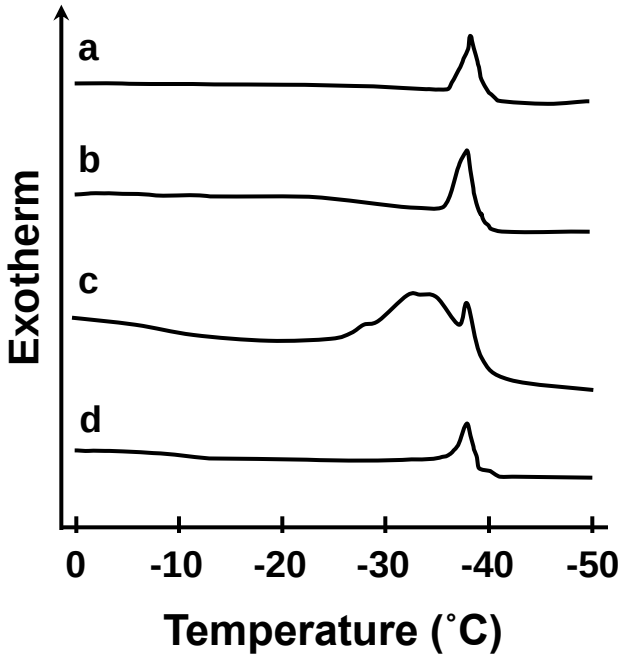

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