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Analysis of supercooling-facilitating (anti-ice nucleation) activity of flavonol glycosides

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ABSTRACT

Deep supercooling xylem parenchyma cells (XPCs) of katsura tree (Cercidiphyllum japonicum) contain four kinds of flavonol glycosides with high supercooling-facilitating (anti-ice nucleation) activities. These flavonol glycosides have very similar structures, but their supercooling-facilitating activities are very
different. In this study, we analyzed the supercooling-facilitating activities of 12 kinds of flavonol glycosides in order to determine the chemical structures that might affect supercooling-facilitating activity. All of the flavonol glycosides tested showed supercooling-facilitating activity, although the magnitudes of activity differed among the compounds. It was clear that the combination of the position of attachment of the glycosyl moiety, the kind of attached glycosyl moiety and the structure of aglycone determined the magnitude of anti-ice nucleation activity. However, there is still some ambiguity preventing the exact identification of features that affect the magnitude of supercooling-facilitating activity.

**Keywords:** flavonol glycosides; supercooling-facilitating (anti-ice nucleation) activity; xylem parenchyma cells; deep supercooling.

**Introduction**

Xylem parenchyma cells (XPCs) of woody plants adapt to subfreezing temperatures by deep supercooling [3,5,6]. In XPCs of katsura tree (*Cercidiphyllum japonicum*), which maintain deep supercooling to -40°C during winter, we have
recently identified four supercooling-facilitating (anti-ice nucleation) flavonol glycosides: kaempferol 7-O-β-D-glucopyranoside (K7Glc), kaempferol 3-O-β-D-glucopyranoside (K3Glc), 8-methoxykaempferol 3-O-β-D-glucopyranoside (8MK3Glc), and quercetin 3-O-β-D-glucopyranoside (Q3Glc) [4]. The supercooling-facilitating activities of these compounds were very high compared with those of previously reported substances [2], suggesting that these compounds play an important role in the mechanism that facilitates the supercooling capability of XPCs [4].

It was also noted in our previous study [4] that although these four flavonol glycosides exhibited very similar structures, their supercooling-facilitating activities fluctuated greatly, ranging from 2.8°C to 9.0°C. These results suggest that minor changes in the structures of flavonol glycosides greatly affect the magnitudes of supercooling-facilitating activity. The results also suggest that other compounds of flavonol glycosides may have effects to facilitate supercooling. In this study, therefore, we analyzed the effects of other flavonol glycosides to facilitate supercooling. We measured the supercooling-facilitating activities of 12 kinds of flavonol glycosides, including two kinds of kaempferol glycosides (with one hydroxyl group at the B-ring), eight kinds of quercetin glycosides (with two hydroxyl groups at the B-ring) and two kinds of myricetin glycosides (with three hydroxyl
groups at the B-ring), in order to determine the chemical structures in flavonol glycosides that affect supercooling-facilitating activity as well as to find flavonol glycosides with higher supercooling-facilitating activity.

**Materials and methods**

We analyzed supercooling-facilitating activities of 12 kinds of flavonol glycosides, kaempferol 3-\(\beta\)-D-rutinoside (K3Rut), kaempferol 7-\(\beta\)-D-galactopyranoside (K7Gal), quercetin 5-\(\beta\)-D-glucopyranoside (Q5Glc), quercetin 7-\(\beta\)-D-glucopyranoside (Q7Glc), quercetin 3’-\(\beta\)-D-glucopyranoside (Q3’Glc), quercetin 4’-\(\beta\)-D-glucopyranoside (Q4’Glc), quercetin 3-\(\beta\)-D-galactopyranoside (Q3Gal), quercetin 7-\(\beta\)-D-galactopyranoside (Q7Gal), quercetin 3-\(\alpha\)-L-rhamnopyranoside (Q3Rha), quercetin 3-\(\beta\)-D-rutinoside (Q3Rut), myricetin 3-\(\beta\)-D-glucopyranoside (M3Glc) and myricetin 3-\(\alpha\)-L-rhamnopyranoside (M3Rha). The chemical structures of these compounds are summarized in Fig. 1. Among these flavonol glycosides, Q5Glc, Q7Glc, Q3’Glc, Q7Gal and K7Gal were organically synthesized in our laboratory in accordance with procedures by Dick and Smith [1]. Two myricetin glycosides were generously
provided by Dr. H. Hara. The other flavonol glycosides were commercial products from Extrasynthèse (Genay, France).

Anti-ice nucleation activities were determined by a droplet freezing assay [4]. Each flavonol glycoside was diluted at a concentration of 1 mg/ml in phosphate buffer (50 mM potassium phosphate, pH 7.0) containing 2 mg/ml of UV-sterilized and lyophilized ice nucleation bacteria, *Erwinia ananas* (Wako Pure Chemical Industries, Osaka, Japan). These samples at 2 µl in each droplet were placed on a copper plate that was floated on a coolant in an alcohol bath (F26; Julabo Labortechnik, Seelback, Germany). The plate was maintained at 0°C for 5 min and then cooled at a rate of 0.2°C/min to -20°C. During the cooling process, the number of frozen droplets was counted every 0.5°C temperature decrement by the naked eye. In total, more than 200 droplets from more than five separated examinations were used for each compound and the percentage of cumulative number of frozen droplets was plotted. The temperature required for freezing of 50% of the total droplets was indicated as INT<sub>50</sub> (ice nucleation temperature for 50% of droplets). The differences in INT<sub>50</sub> between the control solution not containing flavonol glycosides and sample solutions containing each flavonol glycoside were defined as supercooling-facilitating (anti-ice nucleation) activities.
Results and discussion

The supercooling capability of the control solution without addition of flavonol glycoside was -5.2°C in our system as revealed by INT$_{50}$ (Fig. 2). The addition of 1 mg/ml glucose (similar concentration with added flavonol glycosides) also showed INT$_{50}$ at -5.2°C (result not shown). Due to the very low concentration of added solutes, no detectable ice nucleation temperature depression occurred by a colligative effect. The ice nucleation temperatures were depressed by a non-colligative effect in all the cases by the addition of each flavonol glycoside used in this study. These results indicated that all of the flavonol glycosides had supercooling-facilitating activity. However, there were large differences in the magnitudes of supercooling-facilitating activity of the flavonol glycosides (Fig. 2).

**Kaempferol:** The anti-ice nucleation activities of kaempferol glycosides were first examined. Kaempferol is different from other flavonols, the B-ring of aglycone having only one hydroxyl group at the C-4’ position (Fig. 1a). We used K3Rut and K7Gal as kaempferol glycosides. Since two kinds of kaempferol glycosides, K3Glc and K7Glc, that exist in deep supercooling XPCs in katsura tree showed high
supercooling-facilitating activities of 4.0°C and 9.0°C, respectively [4], we expected that other kaempferol glycosides would also have high supercooling-facilitating activities. However, the activities of K3Rut and K7Gal were not higher than those of K3Glc and K7Glc. The activities were 1.7°C for K3Rut (INT<sub>50</sub> = -6.9°C) and 0.4°C for K7Gal (INT<sub>50</sub> = -5.6°C). These results suggested that difference in the kind of attached glycosyl moiety had a great effect on supercooling-facilitating activity even though the position of attachment is the same.

**Quercetin:** The anti-ice nucleation activities of quercetin glycosides were examined next. Quercetin is different from kaempferol, the B-ring having two hydroxyl groups at the C-3’ and 4’ positions (Fig. 1b). Q3Glc has been shown to be present in XPCs of katsura tree and to exhibit supercooling-facilitating activity of 2.8°C [4]. In this study, eight other kinds of quercetin glycosides, Q5Glc, Q7Glc, Q3’Glc, Q4’Glc, Q3Gal, Q3Rha, Q3Rut and Q7Gal, were examined. Among these compounds, the supercooling-facilitating activities of Q7Glc, Q5Glc, Q3’Glc and Q4’Glc were first compared (Fig. 2b-1) because the structural difference in these four compounds is only the position at which glucosyl moiety is attached (Fig. 1b). Q7Glc showed very high supercooling-facilitating activity of 8.9°C (INT<sub>50</sub> = -14.1°C). In contrast, the activity of Q5Glc was only 0.4°C (INT<sub>50</sub> = -5.6°C). Q3’Glc and Q4’Glc showed intermediate magnitudes of supercooling-facilitating
activities of 1.4°C (INT$_{50}$ = -6.6°C) and 3.1°C (INT$_{50}$ = -8.3°C), respectively. These results suggested that even in the case of attachment of the same kind of glycosyl moiety, the position of attachment greatly affects the supercooling-facilitating activity.

In quercetin glycosides, we also compared effects on supercooling of different kinds of glycosyl moiety attached to the same position (Fig. 2b-2). Among the quercetin glycosides, the effects of Q3Gal, Q3Rha and Q3Rut, in which different kinds of a glycosyl moiety are attached at the C-3 position, were compared. Rhamnopyranoside (Q3Rha) and rutinoside (Q3Rut) showed little anti-ice nucleation activities of 0.4°C (INT$_{50}$ = -5.6°C) and 0.1°C (INT$_{50}$ = -5.3°C), respectively, but galactopyranoside (Q3Gal) showed high activity of 7.6°C (INT$_{50}$ = -12.8°C). These results also confirmed that different kinds of glycosyl moiety greatly affected supercooling-facilitating activity in quercetin glycosides similar to kaempferol glycosides (Fig. 2a).

Among the three kinds of quercetin glycosides with different glycosyl moieties at the C-3 position (Fig. 2b-2), the highest supercooling-facilitating activity was observed in the case of attachment of a galactosyl moiety. Therefore, we also examined the effect of the galactosyl moiety on supercooling-facilitating activity when it was attached at the C-7 position of quercetin (Fig. 2b-2). Q7Gal showed
much lower anti-ice nucleation activity than that of Q7Glc (Fig. 2b). Anti-ice nucleation activity of Q7Gal was 2.2°C ($\text{INT}_{50} = -7.4^\circ\text{C}$). These results indicated that the combination of position of attachment of the glycosyl moiety and kind of attached glycosyl moiety is important for supercooling-facilitating activities of quercetin glycosides.

**Myricetin:** We finally examined the effects of myricetin, in which the difference from kempferol and quercetin is that the B-ring has three hydroxyl groups at C-3’, 4’ and 5’ positions (Fig. 1c). We examined the supercooling-facilitating activities of two kinds of myricetin glycosides, M3Glc and M3Rha (Fig. 2c). Supercooling-facilitating activities of these glycosides were low. The levels of M3Glc and M3Rha were 1.3°C ($\text{INT}_{50} = -6.5^\circ\text{C}$) and 0.3°C ($\text{INT}_{50} = -5.5^\circ\text{C}$), respectively. The results suggested that structures of aglycone may affect supercooling-facilitating activity.

To clarify the relationship between structures and supercooling-facilitating activities of flavonol glycosides, we plotted together 12 kinds of quercetin, kaempferol or myricetin glycosides used in this study and three kinds of flavonol glycosides that were identified from the xylem of katsura tree in a previous study [4] in relation to supercooling-facilitating activities (Fig. 3). From Fig. 3, it is clear that
supercooling-facilitating (anti-ice nucleation) activities of the flavonol glycosides are controlled by the combination of position of attachment of the glycosyl moiety, the kind of attached glycosyl moiety and the structure of aglycone. However, features of structures in flavonol glycosides that clearly affect supercooling-facilitating activity were not found.

Further studies, including experiments using different types of ice nucleation substances are currently conducted in our laboratory to elucidate the mechanisms of supercooling by flavonol glycosides.

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References


Figure legends

Fig. 1  
Chemical structures of flavonol glycosides used in this study: (a) kaempferol glycosides, (b) quercetin glycosides, (c) myricetin glycosides.

Fig. 2  
Ice nucleation temperatures of water droplets containing kaempferol glycosides (a), quercetin glycosides (b-1: glucopyranosides, b-2: other glycosides), or myricetin glycosides (c). Effects of 1 mg/ml of flavonols on ice nucleation temperatures of a buffer solution containing 2 mg/ml of ice nucleation bacteria were determined by a droplet freezing assay.

Fig. 3  
Anti-ice nucleation activities of 15 kinds of flavonol glycosides, including 12 kinds of flavonol glycosides used in this study and 3 kinds of flavonol glucopyranosides used in our previous study [4]. Color of symbols shows the kind of aglycone (blue: kaempferol, red: quercetin, yellow: myricetin), and shape of symbols indicates the kind of attached glycosyl moiety (circle: glucose, triangle: galactose, square: rhamnose, diamond: rutinose). Supercooling-facilitating (anti-ice nucleation) activities were calculated from the difference in INT_{50} between
control solution not containing flavonol glycoside and sample solution containing each flavonol glycoside.
Fig. 1 Chemical structures of flavonol glycosides used in this study: (a) kaempferol glycosides, (b) quercetin glycosides, (c) myricetin glycosides.
Fig. 2 Ice nucleation temperatures of water droplets containing kaempferol glycosides (a), quercetin glycosides (b-1: glucopyranosides, b-2: other glycosides), or myricetin glycosides (c). Effects of 1 mg/ml of flavonols on ice nucleation temperatures of a buffer solution containing 2 mg/ml of ice nucleation bacteria were determined by a droplet freezing assay.
**Fig. 3** Anti-ice nucleation activities of 15 kinds of flavonol glycosides, including 12 kinds of flavonol glycosides used in this study and 3 kinds of flavonol glucopyranosides used in our previous study [4]. Color of symbols shows the kind of aglycone (blue: kaempferol, red: quercetin, yellow: myricetin), and shape of symbols indicates the kind of attached glycosyl moiety (circle: glucose, triangle: galactose, square: rhamnose, diamond: rutinose). Supercooling-facilitating (anti-ice nucleation) activities were calculated from the difference in INT$_{50}$ between control solution not containing flavonol glycoside and sample solution containing each flavonol glycoside.
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