Preparation of a Xyloglucan Oligosaccharide Mixture from Tamarind Seed Gum and its Promotive Action on Flower Opening in Carnation Cultivars

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A mixture of xyloglucan oligosaccharides (XGO) was prepared from xyloglucan (XG) of tamarind seed gum by digestion with \textit{Aspergillus} recombinant XG-specific xyloglucanase and subsequent purification by ethanol fractionation. The XGO mixture contained XG7, XG8, and XG9 at the ratio of 1:4:5, which was almost identical to the literature value of constituent subunits ratio of 1.2:3.8:5. The XGO mixture at 1% promoted flower opening in carnation (\textit{Dianthus caryophyllus} L.) cultivars, such as ‘Pure Red’ and ‘Lillian’. On the other hand, there was no effect on other cultivars, such as ‘Collin’, ‘Light Pink Barbara’, and ‘Mule’. Promotion of flower opening by 1% XGO was exhibited earlier than that by glucose or sucrose at 1% in ‘Pure Red’ carnation. Separate application of XG7 or XG9 stimulated flower opening in ‘Pure Red’ carnation, suggesting that all the constituents in the XGO mixture were effective in stimulating flower opening. Based on these observations, the mechanism of action of XGO on carnation flower opening and its future practical use as a flower-opening agent are discussed.

Key Words: carnation, flower opening, tamarind seed gum, xyloglucan, xyloglucan oligosaccharides.

Introduction

The vase life of cut ornamental flowers is the period from flower opening to senescence. It is necessary to slow down both processes to prolong the display time of the flowers. To achieve such a final goal in the flower-opening process, we have recently studied the molecular mechanism of flower opening using cut carnation flowers as a model ornamental (Harada et al., 2010; Morita et al., 2011). Flower opening involves elongation, expansion, and outward bending of petals, which result from the enlargement of petal cells (Evans and Reid, 1988; Kenis et al., 1985; Koning, 1984).

Petal cell enlargement is accompanied by the new formation and regeneration of its components. Several studies have addressed the cell-wall changes during flower opening (de Vetten and Huber, 1990; Yamada et al., 2009). Cell-wall extensibility may be a growth-limiting factor for petal expansion (Yamada et al., 2009). The plant cell wall is a strong fibrillar network arranged to give each cell a solid shape. Two kinds of apoplastic enzymes, xyloglucan endotransglycosylase/hydrolase (XTH) and expansins, are considered to be involved in cell-wall loosening in plants (Carpita and McCann, 2000). XTH catalyzes the transglycosylation of xyloglucan, which results in cleaving and reattachment among xyloglucan chains [xyloglucan endotransglycosylase (XET) activity], and realignment of the xyloglucan chain in different strata when newly synthesized xyloglucan are incorporated (Fry et al., 1992; Nishitani and Tominaga, 1992; Vissenberg et al., 2000). O’Donoghue et al. (2002) showed that XTH activity is associated with flower opening in sandersonia. Breeze et al. (2004) showed that an XTH gene was up-regulated in developing petals of opening \textit{Alstroemeria} flowers. Laitinen et al. (2007) showed that \textit{Gerbera hybrida} had several XTH genes and their expression pattern during the development of ray flowers differed; some genes were up-regulated in the early stage and down-regulated in the late stage of petal development, and others vice versa. Recently, Harada et al. (2011) cloned four cDNAs for XTH (\textit{DcXTH1–DcXTH4}) from petals of opening carnation (\textit{Dianthus caryophyllus} L.) flowers and examined the expression of their corresponding genes in floral and vegetative tissues of carnation plants with...
opening flowers, revealing that DcXTH2 and DcXTH3 are associated with petal growth and development during carnation flower opening.

Based on the preceding investigation, we considered that if we could disturb or inhibit the cell-wall metabolism in the growing petals of opening flowers by applying an excess amount of xyloglucan oligosaccharides (XGO), we would affect the flower-opening process, resulting in a modified display time of the flowers. XGO was shown to inhibit 2,4-D induced elongation in pea stem segments at pseudo-nanomolar concentrations (McDougall and Fry, 1988; York et al., 1984), and to induce ethylene synthesis in persimmon fruit (Cutillas-Iturralde et al., 1998). To test our hypothesis, we planned to assess the effects of XGO on flower opening of carnation, although XGO would need a higher concentration (at millimolar concentrations) to exert its action. Therefore, we first prepared a sufficient amount of XGO from xyloglucan in tamarind seed gum by enzymatic degradation and subsequent purification. Then we examined the effect of XGO on flower opening in carnation and observed that XGO promoted flower opening in some cultivars. This paper provides details of the simple and quick preparation of an XGO mixture from tamarind seed xyloglucan and its promotive effects on flower opening in carnation, with discussion of its possible action mechanism.

Materials and Methods

Preparation of an XGO mixture from tamarind seed gum

The AaXEG2 cDNA for xyloglucanase of Aspergillus aculeatus (Pauly et al., 1999) was amplified by PCR with a set of primers (forward, 5'-GACGAACAGGC CATGGAGCGCCGACGCACT-3'; reverse 5'-TGCTCGT GAGCCGCCGGTGCCACGCACACGG-3') and pBE2113-AaXEG2 binary vector (Park et al., 2004) as a template. The cDNA was subcloned into pET32 vector (Novagen, Merck KGaA, Darmstadt, Germany) at the Nco I and Not I digestion site using an In-Fusion Cloning Kit (Clontech, Takara Bio Inc., Shiga, Japan) and transformed into E. coli [Origami (DE3) pLysS strain; Novagen]. The expression of thioredoxin (Trx) and His-tag fusion xyloglucanase protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), then E. coli was cultivated at 20°C for 3 days. The protein was recovered from E. coli cells by BugBuster Protein Extraction Reagent (Novagen) with Benzonase Nuclease (Novagen). The recombinant xyloglucanase was purified by a HisTrap HP column (GE Healthcare UK Ltd., Buckinghamshire, UK) and desalted by PD-10 column (GE Healthcare). Purification of the protein was confirmed by SDS-PAGE and Western blot analyses.

Powdered tamarind seed gum (TG120) was a generous gift from MRC Polysaccharide Co., Ltd. (Tokyo, Japan). One gram of tamarind seed gum powder was added slowly to about 90 mL water at 50°C with vigorous agitation, which was continued for more than 4 hours to dissolve the powder. After cooling the solution to 25°C, the solution was made up to 100 mL and adjusted to pH 4.5 with acetic acid, and 100 μg xyloglucanase was added to the solution. Finally, two mL toluene was added to the solution to inhibit bacterial growth. The reaction was carried out at 30°C for 2 days. After enzymatic degradation, ethanol was added to the reaction mixture to make 80%, and the resultant mixture was left at 4°C to precipitate undegraded xyloglucan. After centrifugation at 10,000 × g for 20 min, the alcoholic supernatant was recovered. The XGO mixture was finally recovered from the supernatant solution after drying using a rotary evaporator and freeze-dried.

To confirm the uniformity of the XGO mixture, it was subjected to gel filtration through a BioGel P2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) column (1.6 cm i.d. × 90 cm). The XGO was eluted with 20 mM Na-acetate buffer (pH 4.5) at a flow rate of 0.3 mL·min⁻¹. The XGO in the eluate was monitored by the anthrone-sulfuric acid method using as a standard a commercial product of xyloglucan-derived oligosaccharides (O-XGHON; Megazyme International Ireland, Co. Wicklow, Ireland), which is a mixture of XG7, XG8, and XG9. The O-XGHON sample was similarly eluted from the BioGel P2 column as a reference for XG7, XG8, and XG9.

A sample of the prepared XGO was subjected to matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOFMS) analysis. All spectra were recorded in positive reflector mode on a MALDI-TOFMS instrument (autoflex III; Bruker Daltonics, Bremen, Germany). For analysis, 2,5-dihydroxybenzoic acid (DHB) was used as a matrix, sodium trifluoroacetic acid (NaTFA) as an ionization salt and 0.1% trifluoroacetic acid/acetonitrile (2:1, v/v) as a solvent.

Plant materials and determination of flower opening profiles

Cut flowers of carnation (D. caryophyllus L.) cultivars, all belonging to the spray category of carnation flowers, were harvested when the first flower out of five to six flower buds was nearly open at the nurseries of commercial growers in Miyagi and Kagawa prefectures. The flowers were not treated with any flower preservatives, such as STS, after harvest. The carnation cultivars used were ‘Light Pink Barbara’, ‘Pure Red’, ‘Collin’, ‘Mule’ (all from Miyagi Prefecture), and ‘Lillian’ (from Kagawa prefecture). The flowers were transported dry to the laboratory at Kyoto Prefectural Institute of Agricultural Biotechnology in Kyoto prefecture the day after harvest. They were placed in plastic buckets with their cut stem end in water under continuous light from white fluorescent lamps (14 μmol·m⁻²·s⁻¹ PPFD) at 23°C.

The flower-opening process was separated into 6 stages according to Harada et al. (2010): opening stage
1 (Os 1), petals just emerged from buds; Os 2, petals elongated vertically; Os 3, petal clusters expanded; Os 4, outer petals start to reflex (bend outwards); Os 5, outer petals have reflexed; Os 6, fully open flower with outer petals at right angles to the stem. Carnation flowers at Os 1 or Os 2 were used to examine the effect of XGO on flower opening. Samples of 6 or 7 flowers (florets) with 15 or 20 cm-long stem, depending on given experiments, were placed with their cut stem ends in a 50-mL plastic tube with 30 mL test solution. The control solution was distilled water containing a bactericide at 0.1 mL·L⁻¹ Legend-MK (Rohm and Haas, Darmstadt, Germany: a mixture of 5-chloro-2-methyl-1,2-thiazol-3-one and 2-methyl-1,2-thiazol-3-one). Test solutions contained the XGO mixture, glucose or sucrose at 1% in the control solution. As the XGO solution was a mixture of XG7, XG8, and XG9 (Fig. 3), the average molar concentration of 1% XGO solution was estimated to be 7.8 mM, when calculated based on the component ratio of 1 : 4 : 5 and the molecular weights of each component. The 1% solution was 55.5 mM for glucose, and 29.2 mM for sucrose. The flowers were left under the same condition as described above, and the flower-opening process was checked every day and photographed. The control and test solutions were refilled to 30 mL every day. Experiments, in which the effect of 1% XGO was tested (Fig. 4), were repeated 3 times for ‘Pure Red’, ‘Collin’, and ‘Lillian’, and once for ‘Light Pink Barbara’ and ‘Mule’ carnation. The experiment for comparison of XGO with glucose and sucrose was carried out once with 3 replications, each with 5 flowers (Fig. 5).

When the effects of XGO, XG7, and XG9 on flower opening in ‘Pure Red’ carnation were compared, a flower with 5 cm-long stem was placed in a 2.5-mL plastic tube with 2 mL test solution. Test solution was 1% XGO, 0.1% XG7, 0.5% XG7, 0.5% XG9, and the control as described above. XG7 and XG9 were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan): Heptasaccharide Glc₄Xyl₃ (CAS no. 121591-98-8) and Nona-saccharide Glc₄Xyl₃Gal₂ (CAS no. 129865-06-1). The experiment was conducted with 5 flowers per treatment.

To see the effect of XGO treatment on the flower-opening profile of carnation flowers displayed in a vase, bunches of 10 flowers each of ‘Pure Red’ carnation (50–60 buds and florets in total), were placed in a 0.9 L glass jar with 400 mL of the control and 1% XGO solutions. Flower-opening profiles were observed every day and photographed for 14 days.

Statistical analyses were carried out by Mann-Whitney’s U-test or Steel’s multiple range test using an on-line statistical analysis program MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/).

Results

Preparation of an XGO mixture from tamarind seed gum

We prepared a mixture of XGO from tamarind seed gum after degradation with Aspergillus recombinant xyloglucan-specific xyloglucanase and subsequent ethanol fractionation. The yield ranged from 66 to 77% in the repeated experiments, in which 1 g tamarind seed gum was usually used for preparation. The XGO mixture was eluted from the BioGel P2 column as a single peak, and the elution profile was the same as that of the commercial preparation of XGO composed of XG7, XG8, and XG9, which was used as the standard (Figs. 1 and 3).

MALDI-TOFMS analysis gave three peaks corresponding to sodium adduct ions of XG7, XG8, and XG9 [M + Na]+ (Fig. 2). From peak heights, the ratio in the contents of XG7, XG8, and XG9 in the XGO mixture was estimated to be 1 : 4 : 5. In MALDI-TOFMS analysis, two XG8s, XLXG and XXLG (Fig. 3), were not separated.
Promotion of flower opening in ‘Pure Red’ and ‘Lillian’ carnation by the XGO mixture

First, we examined the effects of XGO on flower opening in several carnation cultivars, ‘Pure Red’, ‘Lillian’, ‘Collin’, ‘Light Pink Barbara’, and ‘Mule’. Carnation flowers were treated continuously with 1% XGO for 10 days. Figure 4 shows the time course of flower opening during 10 days and flowering profiles 5 days (‘Pure Red’ and ‘Collin’) or 6 days (‘Lillian’) after the start of the experiment. XGO treatment promoted flower opening in ‘Pure Red’ and ‘Lillian’. In ‘Pure Red’ carnation, the stimulation of flower opening was significant even one day after the start of treatment.

Comparison of the XGO mixture with sucrose and glucose in the promotion of flower opening in ‘Pure Red’ carnation

Figure 5 compares the effect of XGO with that of glucose and sucrose on flower opening in ‘Pure Red’ carnation. All test chemicals were used at 1%. Although there was no significant difference, XGO tended to promote flower opening 2 and 3 days after the start of treatment, and glucose and sucrose did not during these periods. After 5 days, XGO, glucose, and sucrose tended to promote flower opening.

Comparison of the XGO mixture, XG7, and XG9 on flower opening in ‘Pure Red’ carnation

The prepared XGO mixture contained XG7, XG8, and XG9 in the ratio of 1:4:5 as described above. It would be interesting to know whether all three constituents are active in the promotion of flower opening or whether it is a specific constituent(s). We therefore tested the activity of XG7 and XG9 by comparing with XGO. Since 1% XGO solution contains 0.5% XG9 and 0.1% XG7, we tested 0.5% XG9, 0.1% and 0.5% XG7 as well as 1% XGO. Figure 6 shows flower-opening scores among treatments and flower-opening profiles 4 days after the start of treatment. The flower-opening profiles were photographed using 2 typical florets out of the 5 florets used for each treatment. Treatment with 1% XGO and 0.5% XG7 promoted flower opening, but 0.1% XG7 did not. XG9 at 0.5% apparently promoted flower opening, although the promotion was not statistically significant. This observation suggested that XG7, which makes up 10% of the XGO preparation, does not contribute to the promotion of flowering by the XGO preparation, but has the capability to promote flower opening at higher concentrations.

Promotion of flower opening by the XGO mixture in a bunch of ‘Pure Red’ flowers displayed in a vase

To check the potential use of XGO preparations practically as a chemical to modify flower opening, we treated bunches of 10 flowers (50 cm in stem-length) for 2 weeks with or without 1% XGO. Figure 7 compares the flowering profiles of the control and 1% XGO-treated flowers. XGO promoted flower opening in ‘Pure Red’ flowers with 50-cm long stems. On the other hand, senescence, which was observed 2 weeks after the start of treatment in the control and XGO-treated flowers, was almost unchanged between the control and XGO-treated flowers (data not shown).
Discussion

In the present study we developed a simple procedure for preparation of an XGO mixture from tamarind seed gum by enzymatic degradation followed by ethanol fractionation, and revealed its promotive action on flower opening in carnation cultivars, such as ‘Pure Red’ and ‘Lillian’. The XGO preparation was composed of XG7, XG8, and XG9 in the ratio of 1 : 4 : 5. This observed ratio was almost the same as that reported in the literature; i.e., tamarind seed xyloglucan contains these subunit structures in the ratio of XG7 (XXXG) : XG8 (XLXG + XXLG) : XG9 (XLLG) = 12 : 38 : 50 (Yaoi, 2012). At present, XGO preparations are not available commercially in a large quantity at a low price, but they could be supplied, without elaborate and time-consuming purification steps, in the necessary amount when demand arises.

The XGO preparation promoted flower opening in carnation but its action differed depending on the cultivar; i.e., promotion in ‘Pure Red’ and ‘Lillian’ cultivars and no action in ‘Collin’, ‘Light Pink Barbara’, and ‘Mule’ cultivars. We did not further investigate the reason for the lack of action in some carnation cultivars. However, these observations suggest that the action of XGO preparation may differ depending on the flower species as well as the cultivar. Therefore it will be of interest to test the action of the XGO preparation broadly in different ornamental flowers, such as sandarsonia, Alstroemeria and Gerbera hybrida, as mentioned in the Introduction, since the involvement of xyloglucan metabolism in flower opening has been suggested in these flowers (Breeze et al., 2004; Laitinen et al., 2007; O’Donoghue et al., 2002).

Glucose and sucrose are generally thought to act as
an energy source in cut flowers, resulting in promotion of their opening, the latter being degraded to glucose and fructose by invertase. In the present study, the promotion of flower opening by the XGO mixture in ‘Pure Red’ carnation appeared earlier than that by glucose and sucrose (Fig. 5). This observation suggested that the action mechanism of the XGO mixture is different from those of glucose and sucrose; XGO acts without degradation to monosaccharides, thereby not supplying glucose and other monosaccharides as energy sources. Moreover, it was revealed that the action of XGO was not specific to its components; XG7, XG8, and XG9 were all active in the promotion of flower opening (Fig. 6). XG7 at 0.1% (corresponding to 0.94 mM) did not promote flower opening but XG7 at 0.5% (4.7 mM) did, and XG9 at 0.5% (3.6 mM) tended to.

Xyloglucan endotransglucosylase (XET) activity of XTH catalyzes the transglycosylation of xyloglucan, in which one chain of xyloglucan is cleaved and reattached to the non-reducing end of another xyloglucan chain (Fig. 8) (Fry et al., 1992; Nishitani and Tominaga, 1992; Vissenberg et al., 2000). Such a mechanism allows cellulosic microfibrils to undergo transient slippage. XET activity and xyloglucan were able to be simultaneously localized in plant tissues according to fluorescence from wall-bound xyloglucan conjugated with XLLG-SR (sulforhodamine) by the action of XET (Vissenberg et al., 2000). Harada et al. (2011) showed that XTH was actively involved in the expansion growth of petals of carnation flowers during flower opening. Moreover, by an in situ staining experiment of XTH activity using rhodamine-labelled XG9 in growing carnation petals, they showed that XET activity could

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**Fig. 6.** Comparison among the XGO mixture, XG7, and XG9 on flower opening of ‘Pure Red’ carnation. ‘Pure Red’ carnation flowers at Os 1–2 were treated with 1% XGO mixture, 0.1% or 0.5% XG7, and 0.5% XG9 for 4 days. Each treatment was conducted with 5 flowers. A. Flower-opening score. The flower-opening scores are the mean of 5 flowers. Histograms with asterisks are significantly different from the control by Steel's multiple range test (p > 0.05). B. Flower-opening profiles. Two typical flowers were chosen from 5 flowers of respective treatments and photographed.

**Fig. 7.** Promotion by 1% XGO of flower opening in a bunch of ‘Pure Red’ carnation. Bunches of 10 flowers of ‘Pure Red’ carnation at the stage when one out of 5 to 6 buds were open, were treated successively without or with XGO mixture at 1%. The photograph was taken 6 days after the start of the experiment.

**Fig. 8.** Possible action mechanism of XGO on the promotion of flower opening in carnation. The drawing was made according to the figure shown in a text book (Biochemistry and Molecular Biology of Plants, Figure. 2.38; Carpita et al., 2000) with necessary modification. A. During the ordinate process of petal cell growth, XET catalyzes cleavage and reattachment among xyloglucan chains, resulting in transient slippage of cellulosic microfibrils. B. When excess of XGO was present, cleaved xyloglucan polymers were transferred to un-anchored XGO, causing more enhanced slippage of cellulosic microfibrils, eventually resulting in the promotion of flower opening.
transfer cleaved xyloglucan polymers to rhodamine-labeled XG9, which acted as an acceptor substrate. Based on this previous experiment, we speculate that exogenously applied XGO acted as a substrate for XET, that is, an acceptor molecule of cleaved xyloglucan polymers (Fig. 8). Since XGO was not anchored to cellulose microfibrils, the formation of XGO-xyloglucan polymer complexes would cause more enhanced slippage of cellulose microfibrils, which was led by turgor in cells, resulting in the promoted expansion growth of petal cells. This mechanism remains to be confirmed in the future.

Previously, XGO was shown to inhibit 2,4-D induced elongation in pea stem segments (McDougall and Fry, 1988; York et al., 1984). Briefly, XGO was prepared from xyloglucan obtained from suspension-cultured sycamore or rose cells, and was rich in nonasaccharide. From xyloglucan obtained from suspension-cultured sycamore or rose cells, and was rich in nonasaccharide. The active ingredient was nonasaccharide, and heptasaccharide had no activity. XGO was active at concentrations of $10^{-8}$–$10^{-3}$ M. These previous results were different from the present study in which both nona- and heptasaccharide were active at the concentration of $10^{-3}$ M. Roughly speaking, from the point of view of active concentration, XGO might exert plant hormone-like activity in inhibiting auxin-induced elongation of pea stem segments, whereas it might act as a substrate for XTH in promoting flower opening in carnation.

Acknowledgements

We thank Associate Professor Kaname Tsutsumiuchi (Chubu University, Kasugai, Aichi Prefecture) for MALDI-TOFMS analysis of the XGO from tamarind seed gum. We also thank Professor Takahisa Hayashi (Tokyo University of Agriculture, Setagaya, Tokyo) for providing pBE2113-AaXEG2 binary vector. The authors are grateful to the students belonging to the Laboratory of Pomology and Vegetable Crops Science, Nihon University for preparation of XGO.

Literature Cited


