Plant Biotechnology 33, 117–121 (2016)
DOI: 10.5511/plantbiotechnology.16.0217c

Note

A fluorometric assay for quantification of fucoidan, a sulfated polysaccharide from brown algae

Yoshie Yamazaki, Yuko Nakamura, Tatsuo Nakamura*

Graduate School of Environment and Information Sciences, Yokohama National University, Yokohama, Kanagawa 240-8501, Japan
*E-mail: nakamura-tatsuo-tj@ynu.ac.jp Tel & Fax: +81-45-339-4416

Received January 2, 2016; accepted February 17, 2016 (Edited by K. Hiratsuka)

Abstract Brown algae contain a fucose-rich sulfated polysaccharide called fucoidan which is a constituent of the extracellular matrix. In recent years, fucoidan has attracted much attention for its pharmaceutically important biological activities. To develop a superior method for fucoidan quantification, we investigated several cationic dyes with potential interaction with the negatively charged sulfate groups of fucoidan and found that a commercially available fluorescent dye, SYBR Gold nucleic acid gel stain, showed a clear fluorescence enhancement upon mixing with authentic fucoidan. A standard curve generated with the dye and authentic fucoidan showed a linear dynamic range of 0.05–2.5 ng μl^{-1}, and the limits of detection and quantification of fucoidan were 0.025 and 0.075 ng μl^{-1}, respectively. Neither nucleic acids nor alginates, anionic polymers present in brown algae, interfered with the quantification of fucoidan, which is simply extracted with diluted HCl from sporophytes of the brown alga *Saccharina japonica*. The fluorometric microplate method in this study is simple, sensitive, and widely applicable to fucoidan quantification.

Key words: Brown algae, cationic dye, fluorometric measurement, fucoidan, microplate.

Fucoidan is a fucose-rich sulfated polysaccharide mainly found in brown algae of the class Phaeophyceae (Berteau and Mulloy 2003; Pomin and Mourão 2008). It shows a wide range of pharmaceutically important biological activities including anticoagulant (Church et al. 1989; Nishino et al. 1991), procoagulant (Liu et al. 2006; Prasad et al. 2008), anticancer (Riou et al. 1996; Haneji et al. 2005), and antiviral activities (Hoshino et al. 1998; Iqbal et al. 2000). Although many applied studies of the biological activities of fucoidan have been reported, knowledge of its synthesis mechanism and physiological roles in brown algae is limited.

Several methodologies for fucoidan quantification have been reported, and include competitive enzyme-linked immunoassay (ELISA; Irhimeh et al. 2005; Mizuno et al. 2009), sandwich ELISA assay (Tokita et al. 2010), and methylene blue staining of fucoidan immobilized on a filter (Lee et al. 2012). A semiquantitative method using toluidine blue staining of fucoidan separated by agarose or polyacrylamide gel electrophoresis (Pereira et al. 1999) has also been reported. A feature of ELISA methods is that they can be used to quantify specific substructures recognized by each of the antibodies; therefore, the specificity of antibodies affects quantification (Tokita et al. 2010; Torode et al. 2015). Another feature is that the availability of the antibodies directly affects the generality of the methods. Staining methods require commercially available cationic dyes to quantify the sulfate groups, which are important structures for biological activity (Ale et al. 2011). Thus, the ELISA methods and the staining methods are expected to complement each other in quantification of fucoidan. Because fucoidan quantification is an essential procedure for both applied and basic research, development of a quantification method superior to those reported to date would contribute to fucoidan studies. In this study, we developed a simple, sensitive, and widely available fluorometric microplate method for fucoidan quantification using a commercially available fluorescent dye. The method may be applied to extracts simply prepared from small brown algal samples.

Because fucoidan, similar to nucleic acids, is an anionic polymer, several nucleic acid staining dyes were tested for its quantification. An authentic sample of fucoidan from kelp, *Fucus vesiculosus* (Sigma-Aldrich Japan, Tokyo, Japan), was mixed with each dye, including ethidium bromide (Wako Pure Chemical Industries, Ltd., Osaka, Japan), SYBR Green I nucleic acid gel stain (Thermo Fisher Scientific, Waltham, MA, USA), SYBR

Abbreviations: DNase, deoxyribonuclease; DW, dry weight; ELISA, enzyme-linked immunoassay; FW, fresh weight; LOD, limit of detection; LOQ, limit of quantification; RNase, ribonuclease; RSD, relative standard deviation.

This article can be found at http://www.jspcmb.jp/
Published online April 21, 2016

Copyright © 2016 The Japanese Society for Plant Cell and Molecular Biology

Gold nucleic acid gel stain (Thermo Fisher Scientific), or toluidine blue O (Waldeck GmbH, Münster, Germany), and the fluorescence spectra of the solutions were measured. Among the dyes tested, only SYBR Gold stain showed clearly enhanced fluorescence on mixing with fucoidan solution, although the fluorescence spectrum of the solution was slightly different from those of nucleic acids (Figure 1A). The excitation peak was observed at ca. 470 nm, shorter than that of DNA (ca. 495 nm), and the emission peak was at ca. 600 nm, longer than that of DNA (ca. 537 nm). SYBR Gold stain is an asymmetrical cyanine dye that shows a large fluorescence enhancement of approximately 1000-fold upon binding to nucleic acids including double- or single-stranded DNA, and RNA (Tuma et al. 1999). This suggested that fucoidan mimicked single-stranded DNA or RNA in the solution and interacted with the dye to enhance its fluorescence, a phenomenon that could be used for fucoidan quantification without destaining procedures. To the best of our knowledge, there have been no reports regarding the use of SYBR stains for detection of compounds other than nucleic acids.

Further, we searched for optimal conditions yielding a standard curve of fucoidan with a wide linearity range and a low limit of detection (LOD) and quantification (LOQ). Therefore, 50 μl of staining solution containing SYBR Gold stain (with 2,500-, 1,250-, 625-, or 312.5-fold dilution) and 80 mM of buffer [sodium acetate buffer (pH 5.0), sodium phosphate buffer (pH 6.8), or Tris-HCl buffer (pH 7.5 or 8.0)] was mixed with 150 μl of sample solution containing authentic fucoidan (0.125–6 ng μl^{-1}) in 4.88 mM NaCl, and fluorescence was measured to generate a standard curve. A staining solution containing 625-fold diluted SYBR Gold stain and 80 mM Tris-HCl (pH 7.5) was found to be most appropriate, with a standard curve with a linear dynamic range of 0.05–2.5 ng μl^{-1} (R^2=1.00; Figure 1B). LOD (0.025 ng μl^{-1}) and LOQ (0.075 ng μl^{-1}) were calculated to be 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the responses of 10 blanks and S is the slope of the standard curve. Our method successfully generated a standard curve at lower fucoidan concentrations than those by the staining method and the competitive ELISA method. The method that used methylene blue staining of fucoidan blotted on a membrane measured in excess of 1 μg/spot (Lee et al. 2012), while Mizuno et al. (2009) reported a standard curve at fucoidan concentrations above 5 ng μl^{-1} using the competitive ELISA method. On the other hand, the sandwich ELISA method described by Tokita et al. (2010) showed higher sensitivity (LOD, 0.2 ng ml^{-1}) than ours. The standard curve generated with our method showed a 50-fold linear dynamic range, which was wider than any reported to date; 20-fold (Lee et al. 2012), 10-fold (Mizuno et al. 2009), 18-fold (Tokita et al. 2010).

Although we could show the potential value of the

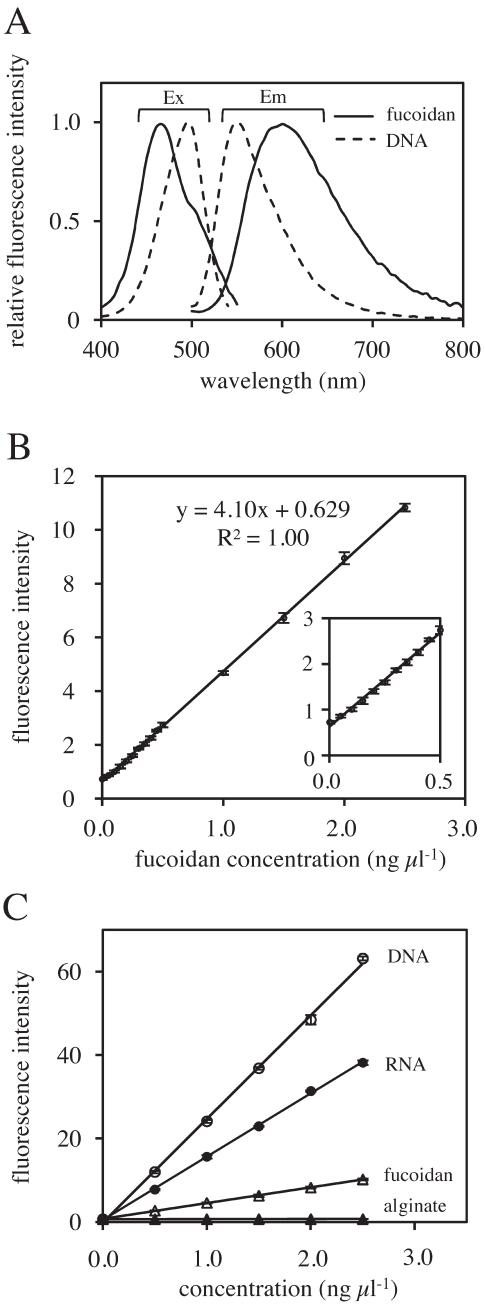

Figure 1. Fluorescence excitation and emission spectra of SYBR Gold stain at 2,500-fold dilution with 2 ng μl^{-1} fucoidan (solid line) or 0.3 ng μl^{-1} DNA (dashed line) (A). Emission spectra were measured on excitation at 470 nm, and excitation spectra on emission at 600 nm using a fluorescence microplate reader (Varioskan Flash, Thermo Scientific). Fucoidan standard curve generated using SYBR Gold stain (B). 150 μl of fucoidan solution with concentrations ranging from 0 to 2.5 ng μl^{-1} in 4.88 mM NaCl was mixed with 50 μl of 625-fold diluted SYBR Gold stain solution in 80 mM Tris-HCl (pH 7.5) in a microplate. Inset shows the standard curve at low fucoidan concentrations. Error bar represents standard deviation (n=3). Comparison of standard curves for DNA, RNA, fucoidan, and sodium alginate prepared with SYBR Gold stain (C). 150 μl of each authentic sample solution in 4.88 mM NaCl was mixed with 50 μl of 625-fold diluted SYBR Gold stain in 80 mM Tris-HCl (pH 7.5) in a microplate. The fluorescence measurements in (B) and (C) were performed on excitation at 470 nm and emission at 600 nm using the fluorescence microplate reader. Error bar represents standard deviation (n=3).

Copyright © 2016 The Japanese Society for Plant Cell and Molecular Biology

stain for fucoidan quantification, brown algae contain compounds that may react with the dye, including DNA, RNA, and alginate, another anionic polysaccharide and the principal constituent of brown algal cells. To investigate the response of these compounds to the dye, we generated standard curves for fucoidan, DNA (plasmid DNA), RNA (total RNA purified from rice leaves), and sodium alginate (300–400 cp; Wako; Figure 1C).

Alginate showed a negligible level of fluorescence enhancement (Figure 1C), approximately 790-fold lower than that of fucoidan. Alginate contained in sporophyte blades can constitute 10–40% of the dry weight (DW) (Davis et al. 2003), generally greater than that of fucoidan, which was 5–20% DW in brown algae of the family Laminariaceae (Davis et al. 2003). However, the negligible level of fluorescence enhancement by alginate indicated that alginate would not interfere with fucoidan quantification, even when a fucoidan-containing fraction was contaminated with the same proportion of alginate as found in brown algal cells.

In contrast, levels of fluorescence enhancement by DNA and RNA were approximately six- and four-fold greater, respectively, than that by fucoidan (Figure 1C). The maximum contents of DNA and RNA in the sporophyte blade of the brown alga *Saccharina japonica* (formerly *Laminaria japonica*), were 0.145% DW and 0.249% DW, respectively (Mizuta et al. 2003). Although these were smaller than the fucoidan contents described above, they prompted our investigation of the effect of contamination by nucleic acids in fucoidan-containing fractions on fucoidan quantification.

Before clarifying this point, we investigated appropriate conditions for extracting fucoidan from brown algae. The extraction procedures were as follows. Approximately 10-mg fresh weight (FW) of a sporophyte blade of *S. japonica* was frozen in liquid nitrogen and ground in a TissueLyser LT (Qiagen, Hilden, Germany; 50 Hz, 2 min) with two 7-mm stainless steel beads in a 2-ml tube. To minimize interference in fluorometry, lipophilic compounds in the sample were removed by rinsing with 1 ml of *n*-hexane, which had been confirmed beforehand not to dissolve fucoidan. After centrifugation of the tube, the *n*-hexane was removed and the pellet was vacuum-dried. To extract fucoidan from the pellet, we used diluted HCl, which had been used in studies reported to date (Chandía and Matsuhiro 2008; Rioux et al. 2007; Tako et al. 1999). We first determined an optimal condition (HCl concentration, temperature, duration of extraction) that maximized fucoidan yield with minimum degradation. The pellet was suspended in 1 ml of diluted HCl (0–50 mM) followed by mixing for 2–24 h at 4°C or 18°C. Following mixing, the tube was centrifuged and 800 μl of supernatant was transferred to a 1.5-ml tube and neutralized with 20 μl of NaOH (0–2000 mM). This was the fucoidan-containing fraction and the fucoidan concentrations were measured as described above. The yield tended to be higher when extracted with 5–25 mM HCl at 18°C (Figure 2A). We then investigated the degree of fucoidan degradation by agarose gel electrophoresis. Because the extraction in higher HCl concentrations resulted in fucoidan degradation (Figure 2B), we considered extraction using 5 mM HCl and mixing for 24 h at 18°C to be the optimal condition.

Using the fucoidan-containing fraction prepared from blades of *S. japonica*, we measured the recovery

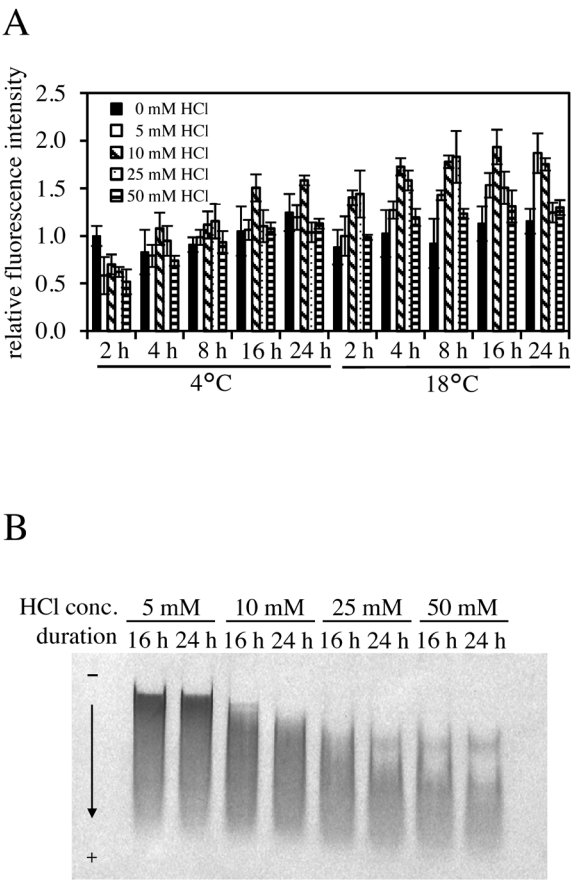

Figure 2. Yield of fucoidan in different extraction conditions (A). HCl concentrations (0, 5, 10, 25, or 50 mM), temperatures (4°C or 18°C), and durations (2, 4, 8, 16, or 24 h) of extraction were examined. Error bar represents standard deviation (n=3). Agarose gel electrophoresis analysis of fucoidan extracted in different extraction conditions (B). 5 μl of each sample solution containing about 1.2 μg of fucoidan, which was extracted with 5–50 mM HCl at 18°C for 16 h or 24 h, was mixed with 2 μl of loading dye containing 0.001% bromophenol blue, 62.5 mM Tris-HCl (pH 6.8), and 40% glycerol, and electrophoresis was performed with 1.2% agarose gel using the Mini-PROTEAN Tetra system (Bio-Rad, Hercules, CA, USA) at 100 V for approximately 40 min in electrophoresis buffer (1× TAE buffer). The gel was stained with 10000-fold diluted SYBR Gold stain in 20 mM Tris-HCl (pH 7.5) for 30 min and rinsed in 20 mM Tris-HCl (pH 7.5) for 30 min. The gel was scanned with a FLA 9000 (FUJIFILM Corporation, Tokyo, Japan) using a 473 nm laser for excitation and LPR (665LP) filter for detection.

Copyright © 2016 The Japanese Society for Plant Cell and Molecular Biology

of fucoidan spiked into fucoidan-containing fractions. Authentic fucoidan (37.5, 150, or 225 ng) was added to the fucoidan-containing fraction and fucoidan contents in each sample were measured using the fluorometric assay. The average recovery rates of spikes (each $n=8$) were 109% (relative standard deviation (RSD), 14%), 100% (RSD, 3.7%), and 97% (RSD, 2.6%), respectively, indicating that components in the fucoidan-containing fraction had little, if any, inhibitory effect on fucoidan quantification.

We then investigated the degree of interference by nucleic acids that may contaminate the fucoidan-containing fractions. Therefore, we measured and compared the fluorescence intensities of fucoidan-containing fractions with or without treatment by deoxyribonuclease I (DNase; Nippongene, Tokyo, Japan) and ribonuclease A (RNase; Nacalai Tesque, Kyoto, Japan; Figure 3). When the fucoidan-containing fractions were spiked with certain amounts of DNA and RNA, their fluorescent intensities increased. When the spiked solutions were treated with both DNase and RNase, the fluorescence level decreased to the level of the fucoidan-containing fraction with no spikes. There was no significant difference between the fluorescence levels of the fucoidan-containing fractions with or without enzyme treatment (Student's t test, $p>0.05$). These results suggested that nucleic acid content in fucoidan-containing fractions would be negligible and accordingly would not interfere with fucoidan quantification.

In summary, we have described a simple, sensitive, and widely applicable fluorometric microplate assay for fucoidan that can be used for analysis of simple extracts from small samples of brown algal tissue.

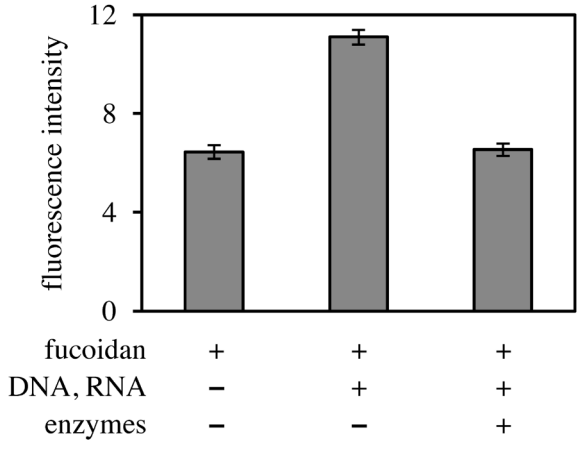

Figure 3. Influence of nucleic acids on fucoidan quantification. 27.5 μl of fucoidan-containing fraction prepared as described above was mixed with or without DNA (230 ng) and RNA (500 ng), and treated with or without DNase (2 unit) and RNase (10 μg) in 100 μl buffered (pH 7.9) solution. Error bar represents standard deviation ($n=8$). The fluorescence was measured as described above.

Acknowledgement

The authors are grateful to Dr. Masahiro Notoya (formerly at Tokyo University of Marine Science and Technology) for providing the kelp sporophytes and for advice.

References

Ale MT, Mikkelsen JD, Meyer AS (2011) Important determinants for fucoidan bioactivity: A critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. *Mar Drugs* 9: 2106–2130

Berteau O, Mulloy B (2003) Sulfated fucans, fresh perspectives: Structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* 13: 29R–40R

Chandía NP, Matsuhiro B (2008) Characterization of a fucoidan from *Lessonia vadosa* (Phaeophyta) and its anticoagulant and elicitor properties. *Int J Biol Macromol* 42: 235–240

Church FC, Meade JB, Treanor RE, Whinna HC (1989) Antithrombin activity of fucoidan. The interaction of fucoidan with heparin cofactor II, antithrombin III, and thrombin. *J Biol Chem* 264: 3618–3623

Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. *Water Res* 37: 4311–4330

Haneji K, Matsuda T, Tomita M, Kawakami H, Ohshiro K, Uchihara JN, Masuda M, Takasu N, Tanaka Y, Ohta T, et al. (2005) Fucoidan extracted from *Cladosiphon okamuranus* Tokida induces apoptosis of human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. *Nutr Cancer* 52: 189–201

Hoshino T, Hayashi T, Hayashi K, Hamada J, Lee JB, Sankawa U (1998) An antivirally active sulfated polysaccharide from *Sargassum horneri* (TURNER) C.AGARDH. *Biol Pharm Bull* 21: 730–734

Iqbal M, McCauley JW, Flick-Smith H (2000) Interactions of bovine viral diarrhoea virus glycoprotein E^{rns} with cell surface glycosaminoglycans. *J Gen Virol* 81: 451–459

Irhimeh MR, Fitton JH, Lowenthal RM, Kongtawelert P (2005) A quantitative method to detect fucoidan in human plasma using a novel antibody. *Methods Find Exp Clin Pharmacol* 27: 705–710

Lee JM, Shin ZU, Mavlonov GT, Abdurakhmonov IY, Yi TH (2012) Solid-phase colorimetric method for the quantification of fucoidan. *Appl Biochem Biotechnol* 168: 1019–1024

Liu T, Scallan CD, Broze GJ Jr, Patarroyo-White S, Pierce GF, Johnson KW (2006) Improved coagulation in bleeding disorders by Non-Anticoagulant Sulfated Polysaccharides (NASP). *Thromb Haemost* 95: 68–76

Mizuno M, Nishitani Y, Tanoue T, Matoba Y, Ojima T, Hashimoto T, Kanazawa K (2009) Quantification and localization of fucoidan in *Laminaria japonica* using a novel antibody. *Biosci Biotechnol Biochem* 73: 335–338

Mizuta H, Ogawa S, Yasui H (2003) Phosphorus requirement of the sporophyte of *Laminaria japonica* (Phaeophyceae). *Aquat Bot* 76: 117–126

Nishino T, Aizu Y, Nagumo T (1991) The influence of sulfate content and molecular weight of a fucan sulfate from the brown seaweed *Ecklonia kurome* on its antithrombin activity. *Thromb Res* 64: 723–731

Pereira MS, Mulloy B, Mourão PAS (1999) Structure and anticoagulant activity of sulfated fucans Comparison between

Copyright © 2016 The Japanese Society for Plant Cell and Molecular Biology

the regular, repetitive, and linear fucans from echinoderms with the more heterogeneous and branched polymers from brown algae. *J Biol Chem* 274: 7656–7667

Pomin VH, Mourão PAS (2008) Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology* 18: 1016–1027

Prasad S, Lillicrap D, Labelle A, Knappe S, Keller T, Burnett E, Powell S, Johnson KW (2008) Efficacy and safety of a new-class hemostatic drug candidate, AV513, in dogs with hemophilia A. *Blood* 111: 672–679

Riou D, Colliec-Jouault S, Pinczon du Sel D, Bosch S, Siavoshian S, Le Bert V, Tomasoni C, Sinquin C, Durand P, Roussakis C (1996) Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. *Anticancer Res* 16(3A): 1213–1218

Rioux LE, Turgeon SL, Beaulieu M (2007) Characterization of polysaccharides extracted from brown seaweeds. *Carbohydr Polym* 69: 530–537

Tako M, Nakada T, Hongou F (1999) Chemical characterization of fucoidan from commercially cultured *Nemacystus decipiens* (Itomozuku). *Biosci Biotechnol Biochem* 63: 1813–1815

Tokita Y, Nakajima K, Mochida H, Iha M, Nagamine T (2010) Development of a fucoidan-specific antibody and measurement of fucoidan in serum and urine by sandwich ELISA. *Biosci Biotechnol Biochem* 74: 350–357

Torode TA, Marcus SE, Jam M, Tonon T, Blackburn RS, Hervé C, Knox JP (2015) Monoclonal antibodies directed to fucoidan preparations from brown algae. *PLoS ONE* 10: e0118366

Tuma RS, Beaudet MP, Jin X, Jones LJ, Cheung CY, Yue S, Singer VL (1999) Characterization of SYBR Gold nucleic acid gel stain: A dye optimized for use with 300-nm ultraviolet transilluminators. *Anal Biochem* 268: 278–288

Copyright © 2016 The Japanese Society for Plant Cell and Molecular Biology