Ratiometric Fluorescent Safranin-O Staining Allows the Quantification of Lignin Contents In Muro

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Running head: *In muro* ratiometric quantification of lignin with safranin-O

Abstract

In some specific vascular plant tissues, lignin can impregnate the entire cell wall to make it more rigid and hydrophobic. Different techniques have been developed in the past years to make possible the quantification of this polyphenolic polymer at the organ or tissue level but difficulties of access to the cellular level remain. Here we describe an approach based on ratiometric emission measurements using safranin-O and the development of a macro adapted for the FIJI software which makes possible the quantification of lignin in three different layers of the cell wall on images captured on a fluorescent confocal microscope.

Key words:

Lignin quantification, safranin-O, cell wall, segmentation, confocal microscopy

1 Introduction

Lignin is a major component of plant cell walls present in some specialized tissues of vascular plants. This polyphenolic polymer is mainly derived from the polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols differing in their degree of methoxylation. After their incorporation, these three *p*-hydroxycinnamyl alcohol precursors (monolignols) give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively. Lignin is responsible for water transport in the xylem vessel elements and confers strength to cell walls contributing to the general mechanical support necessary for plant upright growth [1]. Studies on lignin biosynthesis are motivated by the need to understand its role in plant physiology but also by the increasing use of biomass in agro-industry [2].

A good understanding of lignin metabolism necessarily requires the use of techniques allowing their quantification. Two main "wet chemistry" approaches are commonly used for quantifying lignin, depending upon the plant species and more importantly, whether the tissues contain more or less quantities of lignins. Thus, the method using the solubilization of lignins by acetyl bromide [3, 4] is well adapted to herbaceous species whereas the gravimetric Klason method [5] is more suitable for woody species. There are also different methods to determine the H, G, and S subunit composition and the S/G ratio of lignins. Historically, thioacidolysis [6] and nitrobenzene oxidation [7] methods were used, but they are now gradually replaced by pyrolysis coupled with GC/MS [8] or NMR [9].

These approaches provide relevant information on the different tissues/organs studied but their use leads to irreversible tissue destruction. It is therefore not possible to obtain information concerning lignin quantities and compositions at the cellular level. Therefore, histochemical approaches were also developed such as phloroglucinol staining (Wiesner reaction), which specifically reveals the cinnamaldehyde functions of S and G unit-derivatives. In this case, the intensity of the coloration will depend on the quantity of the red chromogen produced by the reaction [10]. The Maüle test, on the other hand, uses potassium permanganate and hydrochloric acid to transform guaiacyl and syringyl residues into catechols, which then give rise to orange-brown quinones in the case of G lignins and purple-red quinones for G-S lignins [11]. Other approaches exploit the autofluorescence of lignins [12] or the possibility to visualize them by using fluorescent compounds such as auramine [13] and acriflavine [14]. All these techniques can therefore provide relatively detailed spatial information, but without offering the possibility to produce quantitative data.

Safranin-O has an interesting potential as a fluorescent probe [15]. Indeed, the emission spectrum of safranin-O depends on the amount of lignin present in the cell wall (Fig. 1). When present in high amounts, the emission will be more intense in the red part. The emission spectrum (at an excitation of 488 nm) always shows a maximum at 568 nm. Before this value, the aspect of the graph is invariable whatever the amount of lignin. On the other hand, in the orange/red area between 568 and 600 nm, it is dependent on the cell wall environment. Indeed, emission values are higher in the lignified tissues (xylem) compared to the non-lignified tissues. Based on these observations, our group developed an approach including a ratiometric method allowing the relative quantification of lignins by comparing the emissions between 540-560 nm (stable portion) and 570-600 nm (variable portion) [16]. In this chapter, we first describe the methodology allowing the quantification of lignin at the cellular level following confocal microscopy acquisition. In a second step, we demonstrate the possibility to apply a segmentation approach [17] to subdivise the cell wall on these images in an automatic way in order to obtain information on lignification in the cell corners, the compound middle lamella and the secondary cell walls

2 Materials

2.1 Plant growth

1. Seeds of *Arabidopsis thaliana*
2. Pots filled with potting soil.

3. Growth chamber in which temperature and artificial light variations are possible.

2.2 Sample preparation

1. 50% and 70% (v/v) ethanol solutions.
2. Agarose solution at 4% (w/v) in ultrapure water.
3. 0.2% safranin-O in a 50% (v/v) ethanol solution kept in the dark. The solution can be stored for several months at -20°C.
4. Vibroslicer (e.g. VT-1000S, Leica, Wetzlar, Germany) for cutting sections to a thickness of 80 μm.

2.3 Image capture and analysis

1. A confocal laser scanning microscope. We used a Nikon A1R confocal (Nikon, Tokyo, Japan) equipped with a 60x/1.4 aperture oil immersion objective (Plan APO VC) and the NIS Element AR3.0 software.
2. FIJI v2.0 [18] . This can be downloaded at https://fiji.sc
3. The associated FIJI macro developed for the quantification of fluorescence. This can be downloaded at https://zenodo.org/record/5775647#. YbiaNi17RhE.

3 Methods

3.1 Plant growth

Arabidopsis thaliana seeds were stratified during 72 h at 4 °C in the dark. Plants were grown in a GroBank chamber (CLF Plant Climatics GmbH, Wertingen, Germany) under 12 h light (100 PAR light intensity) at 22 °C and 12 h dark at 20 °C for 6 weeks. The photoperiod was then changed to 16 h light, 8 h dark and the floral stems were cut when they reached a size of 30 cm.

3.2 Sample preparation

1. Collect floral stem samples of 0.5 cm length
2. Melt the 4% agarose solution and maintain it at 55°C. Pour 1.5 mL of this solution in enough wells of a 48-well cell culture plate. Let the agarose cool down until it becomes thicker but still stays liquid and place the plant fragment in the center of each well with tweezers. Maintain in a vertical position until the agarose hardens.

3. Leave the plate on the bench for another 10 minutes and then unmold with a thin spatula. Cut the basis of the agarose perfectly perpendicular to the fragment with a razor blade. Glue the agarose-embedded fragment on the specimen holder with cyanoacrylate glue. On the Leica VT-1000S vibroslicer, it is possible to place up to three samples on one holder.
4. Cut 80 µm thick fragments with the vibratome. The speed and the frequency depend on the tissue. For a fragment taken at the base of the floral stem, use a speed of 0.4 mm/s (position 6 on the Leica model) and a frequency of 70 Hz (position 7).
5. Place the fragments in a 48-well cell culture plate containing 1 mL 50% (v/v) ethanol solution.

3.3 Sample staining

1. When all the sections are done, replace the ethanol by a fresh or daily thawed 0.2% safranin-O (w/v) solution and incubate for 10 min on a microplate shaker in the dark at room temperature. The shaking intensity should be high enough to allow good mobility of the fragments in the liquid.
2. Remove all safranin-O solution and replace by 50% ethanol. Incubate with shaking for 10 min in the dark. Remove the solution and rinse twice with the same parameters during 15 min in ultrapure water.
3. Mount sections in water on a glass slide and place a coverslip.

3.4 Image capture

1. Perform the acquisition on the tissues by using 3 independent channels. The first is on autofluorescence excitation at 405 nm, and emission at 450/500 nm), the second is on the green channel with excitation at 488 nm and emission at 530-560 nm. The third is on the red channel with excitation at 561 nm and emission at 570-600 nm. All tracks must be acquired sequentially and parameters must be kept constant for all acquisitions.
2. Select a flat region and adjust your focus to perform images between 6 and 9 µm deep from the surface.

3.5 Image analysis

1. Crop the sharp area of the images acquired in 3.4
2. Treat images using FIJI v2.0 and the associated homemade macro developed for this method. First open the Fiji app, open the macro and finally the image cropped in 1. Run the macro and follow the instructions. The macro includes 2 steps:

2.1 First, a ratio image is generated as previously described [16] (Fig. 2). The result appears as a ratiometric image with a color range between 0 (purple) and 2 (red). The intensity ratio values also appear in the results window and can be used for quantification.

2.2 Combining safranin fluorescence and lignin autofluorescence information, the ratio image obtained in 3.5.1.1 is then segmented, as previously described [17], into 3 cell walls regions: the cell corner (CC), the compound middle lamella (CML) consisting of the middle lamella and the primary cell wall, and the secondary cell wall (SCW). (Fig. 3).

4 Notes

1. The use of a vibratome ensures regular homogenous sectioning of the plant tissues. However, hand sectioning with a sharp razor blade can also be performed. The sections should then be checked under the microscope and only the most fine and regular ones should be selected for the safranin-O staining.
2. If using a vibratome, the agarose solution must be used within the week. In our protocol, we suggest to use multi-well cell culture plates but it is also possible to use 1.5 mL microtubes. Their bottom conical part should first be cut-off and placed inside the rest of the tube. Both parts are then sealed with a piece of Parafilm®. The melded agarose can then be poured in the tube and the sample placed in the center. When the gel has cooled down, it can be unmolded by pressing on the bottom conical portion of the tube.
3. The 0.2% safranin-O in a 50% (v/v) ethanol solution can be stored for several months at -20°C. When the solution is thawed, it should be stored in the dark and used within 24 hours.
4. The samples must be stained and processed in the same day in order to be compared. Each characterization of mutants for example must be done with the corresponding WT.
5. When using the confocal microscope, the focus is more accurate when using the red channel.
6. Accurate quantification should be performed by analyzing at least 3 different plants on which 5 sections were performed and at least 2 images captured per section (total = 30 images)
7. The segmentation macro also allows access to the intensity ratio values in the three layers of the cell wall. It is therefore possible to perform accurate quantification and statistics on lignin amounts.

Figure legends

Figure 1: Average safranin-O spectra (with excitation at 488 nm) of cell walls from different flax (*Linum usitatissimum*) tissues containing low amounts (bast fiber and cambial cell walls) or high amounts (xylem cell walls) of lignins.

Figure 2: Local lignin content determination on Arabidopsis floral stems (wild type and mutants) using safranin O fluorescence-based method. A: Green and red channels correspond to safranin fluorescence. The ratiometric image was obtained by applying the specific FIJI macro on both images. B: The normalized safranin intensity ratios (± SD) are represented (WT=100%). The quantification was performed on interfascicular fibers. Means (± SD) with an asterisk are significantly different according to Student t-test (p-value < 0.05, n = 3). WT: wild type; *prx72*: Salk_136893 mutant; *gaut9*: SALK_040287 mutant.

Figure 3: Automatic segmentation of different cell wall zones. Three masks were applied to images of Arabidopsis flowering stem cross-sections stained with safranin-O and observed by confocal laser scanning microscopy. Green and red channels correspond to safranin-O fluorescence. WCW: whole cell walls; SCW: secondary cell walls; CML: compound middle lamella; CC: cell corner.

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