Note

Image analysis of stress-induced lignin deposition in *Arabidopsis thaliana* using the macro program LigninJ for ImageJ software

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Abstract   In vascular plants, lignin is deposited during morphogenesis but also under stress conditions. Assessing the degree of stress-induced lignin deposition is complicated because it occurs locally and irregularly in plant tissues. In this study, we developed a macro program, LigninJ, for the open-source software ImageJ to automatically and efficiently determine areas and levels of lignification after Wiesner (phloroglucinol-HCl) staining. We used the CIELAB color space for detection of red color following the Wiesner reaction. In addition, LigninJ has a function for adjusting the background level and its white balance to reduce biases that are inherent to individual color images. Furthermore, LigninJ can be used for batch analyses of multiple images, taking about 2 s per image. In this study, we analyzed wound-induced lignin deposition in cotyledons of the *Arabidopsis thaliana* ecotypes Landsberg *erecta* and Columbia and assessed ectopic lignin depositions in roots of *lignescence* (*lig*) mutants of *Arabidopsis*. Our results confirmed that this method is efficient for evaluating the degree of stress-induced lignin deposition.

Key words: *Arabidopsis thaliana*, CIELAB color space, ImageJ macro, stress-induced lignin deposition, Wiesner reaction.

In vascular plants, lignin is predominantly deposited in the xylem and in phloem fibers, and it is important for stability and structural integrity. However, lignin is also deposited as a response to stressors such as pathogen invasion and wounding (Liu et al. 2018). Stress-induced lignin deposition may also occur ectopically due to various mutations or chemical treatments. For instance, the temperature-sensitive *Arabidopsis thaliana* mutant *lignescence* (*lig*) accumulates ectopic lignin in its roots at restrictive temperature conditions through a defect of uridine diphosphate-*N*-acetylglucosamine and subsequent inhibition of protein *N*-glycosylation and induction of endoplasmic reticulum stress (Nozaki et al. 2012). Evaluation of the degree of stress-induced lignin deposition is complicated because it occurs locally and irregularly in plant tissues. The Wiesner (phloroglucinol-HCl) reaction has been used as a simple and semi-specific method for staining deposited lignin to produce red coloration (Nakano and Meshitsuka 1992). The preferred targets of the Wiesner reaction were suggested to be *O*-4-linked coniferyl and sinapyl aldehydes in lignifying cell walls (Pomar et al. 2002). Microscopic photographs taken after the Wiesner reaction have been used to determine the level and area of lignification of tracheary elements (TEs) in a xylogenic culture system of *Zinnia elegans* (Takeuchi et al. 2013; Tokunaga et al. 2005, 2006). Tokunaga et al. (2005, 2006) used gray-scale images and Scion Image software (Scion Corp.) by manually tracing the projected cell areas to quantify lignification of TEs stained by the Wiesner reaction. Takeuchi et al. (2013) identified areas of lignin deposition in TEs and extracellular space stained with the Wiesner reaction using color images and the open-source software ImageJ (Rasband 1997–2020). In their study, color images were separated by applying RGB stacks, and the G stack was used to detect lignin, based on the predominance of red color in the G stack. However, coloration of the selected areas had to be confirmed by visual examination because the selected areas included not only red regions but also other dark areas.

In the present study, we developed the macro program LigninJ for ImageJ to automatically and efficiently determine areas and levels of lignification after Wiesner staining. ImageJ includes a function

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that converts color images to the CIELAB color space (McLaren 1976). The CIELAB color space comprises three components, namely the lightness value ($L*$), the green-red component ($a*$), and the blue-yellow component ($b*$). Following conversion to the CIELAB color space, the $a*$ component can be used in LigninJ to detect red coloration after Wiesner staining. In addition, this macro program includes a function for automatic adjustment of the background level and of its white balance to reduce biases that are inherent to individual images. Furthermore, our macro program facilitates batch analyses of multiple images in one directory, taking about 2 s per image, and it stores results and data on the analytic processing steps.

Using LigninJ, we analyzed wound-induced lignin deposition in cotyledons of the *A. thaliana* ecotypes Landsberg *erecta* (L*er*) and Columbia (Col) and ectopic lignin deposition in roots of *lig* mutants. Our results confirmed that this method is effective for assessing the degree of stress-induced lignin deposition.

For analysis of wound-induced lignin depositions in cotyledons, seedlings of two *A. thaliana* ecotypes (L*er* and Col) were grown aseptically on a horizontal germination medium solidified with agar (GMA) in plastic dishes of 9 cm diameter under continuous lighting (40–60 μmol m^{-1} s^{-1}) at 22°C following one day of a 4°C treatment. The GMA was Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 10 g l^{-1} sucrose buffered to pH 5.7 with 0.5 g l^{-1} 2-(*N*-morpholino)ethanesulfonic acid and solidified with 10 g l^{-1} agar (TC-6; Ina Food Industry Co., Ltd., Ina, Japan). Cotyledons of seedling cultivated for 10 days were cut across the center of the cotyledon using small sterile scissors, the distal parts were removed and the basal parts were fixed at zero, one, two, and three days after cutting using 90% ethanol and were stored at 4°C.

For analysis of ectopic lignin deposition in roots, seedlings of Col background *lig* mutants and of wild-type Col were subjected to a 4°C treatment for three days after which they were grown vertically on GMA (15 g l^{-1} agar) at 18°C for five days and subsequently at 28°C for three days. Seedlings were harvested at zero, one, two, and three days after the transition to 28°C and were then fixed in 90% ethanol and stored at 4°C.

Fixed samples were hydrophilized using an ethanol concentration series, after which they were mounted on plastic frames taped to glass microscopy slides and treated with 1% (w v^{-1}) phloroglucinol and 20% (w v^{-1}) HCl to induce the Wiesner reaction (Nakano and Meshitsuka 1992). The samples were then covered using coverslips, and edges were sealed with wax.

Within 30 min after lignin staining, the preparations were examined, and images were captured using a light microscope (Labophot-2, Nikon, Tokyo, Japan) and a digital camera (DXM1200, Nikon). The objective lens

was protected against HCl gas with a coverslip or cling film (Saran Wrap, Asahi Kasei Co., Tokyo, Japan). The condenser of the light microscope was fully raised and opened, the white balance of the digital camera was adjusted, and the background level of the digital camera was adjusted to 90% saturation. Images were captured at a resolution of 1280×1024 pixels. Captured images were analyzed using the LigninJ macro program (Supplementary Figure S1) for ImageJ (version 1.52r). This macro program facilitates adjustment of background levels and white balance of an RGB image, CIELAB stacking, selection of lignified areas in the $a*$ stack image, measurement and record of selected areas, and mean values of $L*$, $a*$, and $b*$ components. A description of the practical application of LigninJ is shown below. Samples of microscopic pictures, the LigninJ macro program file and the macro file of Microsoft Excel are provided from the author's website (http://bio.sci.ehime-u.ac.jp/morphol/SatoLab).

1. Save a set of color images (e.g., in JPEG or TIFF format) at the same magnification in one working directory.

2. Open an image of a microscale or an image including a scale bar at the same magnification as the sample pictures in ImageJ, and calculate the length of a known distance in pixels. For instance, use the "straight line" function to produce a line of known distance, open the "Analyze" menu and select "Set Scale…", enter "known distance", enter "Unit of length", and confirm a length of 100 μm in pixels.

3. Execute the LigninJ macro program as indicated below. Enter the macro program file named "LigninJ.ijm" in the "plugins" directory of ImageJ, restart ImageJ, and execute LigninJ from the "Plugins" menu. Optionally, open the macro program file by drag-and-drop on the ImageJ panel, and execute the macro from the "Macros" menu. The ImageJ version should be 1.52q or later.

4. Select the directory containing the sample images.

5. Set the "Known distance" and "Unit" (the default setting is 100 μm), "Pixels of known distance" (default is 73.9), lower threshold of the $a*$ value (default is 20), and the lower size limits of the lignified area (default is 20 μm^2) in the "Parameter" dialog box, and click "OK". These values should be adjusted to the respective project.

6. All images in the directory will be analyzed. Two processed images with background and its white balance adjustment (file name_1.jpg; Figure 1B, E) and red color selection with serial numbers (file name_2.jpg; Figure 1C, F) from the original images (Figure 1 A, D), a "Summary.csv" file including lignified area data corresponding to individual images, and a "Results.csv" file with data on lignified areas and their mean values in $L*$, $a*$, $b*$ stacks will be saved in the same
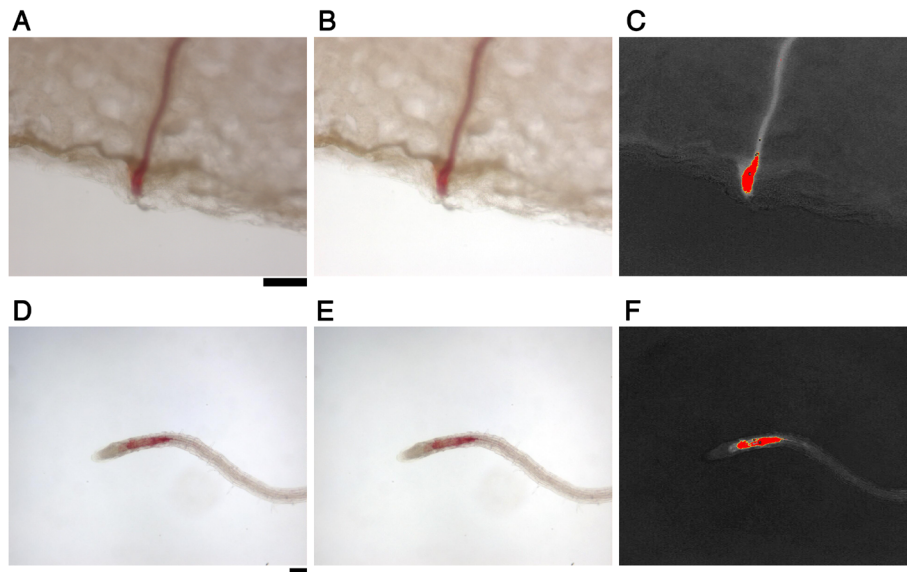
Figure 1.   Pathway of analyses on lignified areas using LigninJ. A–C, A cotyledon of L*er* cultivated for three days after cutting; D–F, *lig* mutants grown at 28°C for two days; A and D, original captured images; B and E, images after adjusting background level and white balance; C and F, *a** stack images showing red color selection with serial numbers. The scale bar indicates 100 μm.
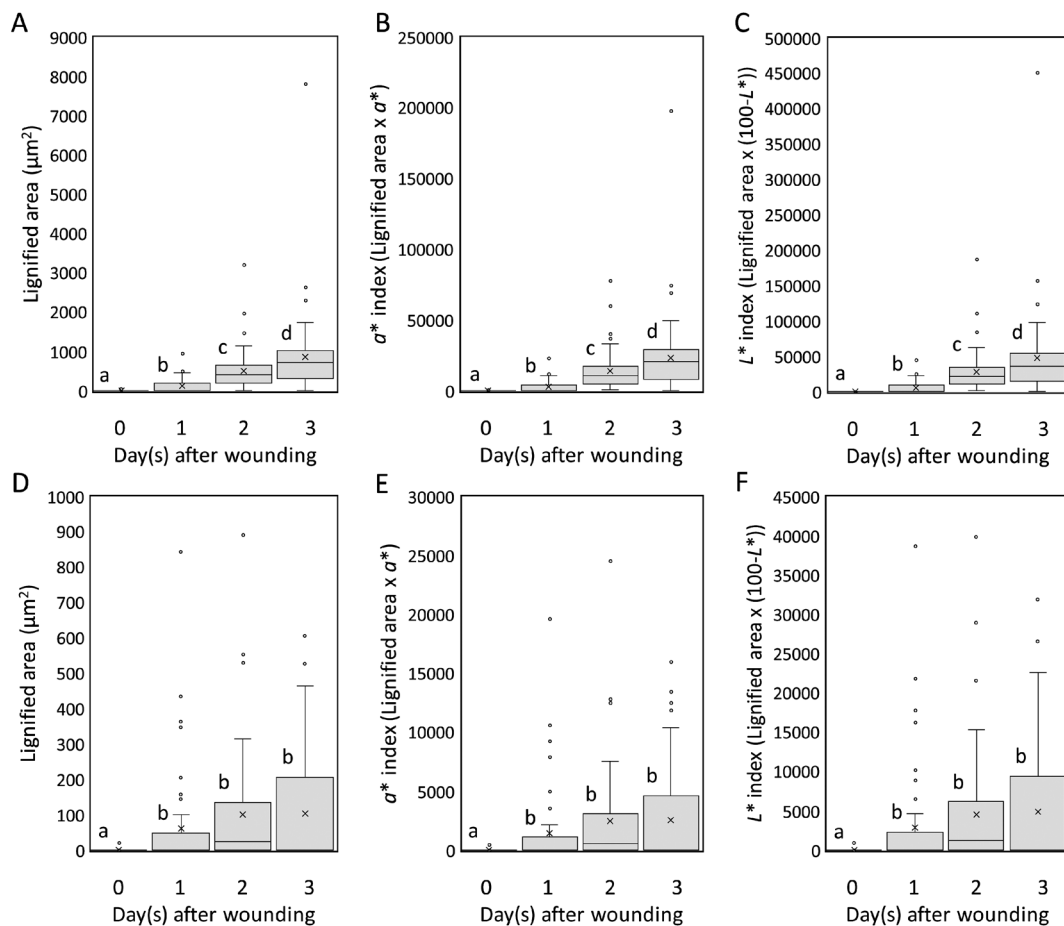

Figure 2.   Analysis of lignified area and *a** and *L** indexes in wounded cotyledons of *A. thaliana*. Box plots show lignified areas (A, D), *a** index (B, E), and *L** index (C, F) of L*er* (A–C) and Col (D–F); the respective average value is indicated by an ×; $n=52$–54. Lower-case letters (a–d) indicate significant differences between data groups in each graph (two-sided Wilcoxon rank sum test; A–C: $p<0.05$; D–F: $p<0.001$).
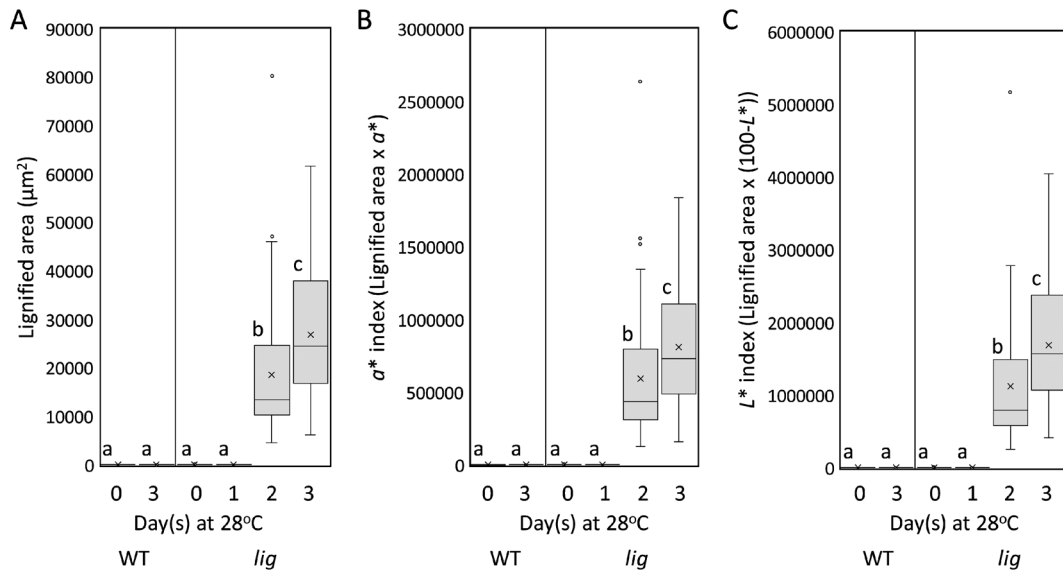
Figure 3.   Analysis of lignified area and *a** and *L** indexes in roots of Col (wild-type; [WT]) and *lig* mutants grown at 28°C. Box plots show lignified areas (A), *a** index (B), and *L** index (C); the respective average value is indicated by an ×; *n*=48–61). Lower-case letters (a–c) indicate significant differences between data groups in each graph (two-sided Wilcoxon rank sum test, *p*<0.001).

directory.

7. Lignified area is defined as "Total Area" value in the "Summary.csv" file. In the current study, the *a** index to indicate lignification levels was defined as the multiplication product of "Area"×*a** in the "Results.csv" file. The *L** index for indicating other lignification levels was also defined as the multiplication product of "Area"×(100−*L**) because the maximum *L** value is 100 (white) and the minimum *L** value is 0 (black).

8. Data in the "Results" sheet of the "Result.csv" file can be transferred and converted to the "Results Calculation" sheet containing *a** and *L** values by using Microsoft Excel to open the file "Results_calculation_excel_macro.xlsm" (Supplementary Figure S2) and running the "Results_calculation" macro of Microsoft Excel on the "Results" sheet of the "Result.csv" file.

9. As a supplementary explanation, "Total Area" in "Summary.csv" did not include holes in the selected areas. "Area" in "Results.csv" includes holes and was used to calculate *a** and *L** indexes. Regarding pictures with no selected lignified area, a zero was shown for "Total Area" in the "Summary.csv", and the area of one pixel for "Area" and zero for "Mean" were used in the "Results.csv" file as a specification of the macro.

All replicates were biological replicates. We cultivated about 50 *A. thaliana* seedlings for each cultivation period and collected sufficient samples for statistical analyses. All data analyses were performed in R 3.5.2 (R Core Team 2019) using R commander 2.5-1 (Fox 2005). A Shapiro–Wilk test confirmed that all data significantly deviated from normal distribution, thus we used non-parametric two-sided Wilcoxon rank sum tests for subsequent analyses.

The analysis pathway of lignified areas using LigninJ macro is shown in Figure 1. The macro facilitates selection and measurement of only newly lignified areas in the veins of cotyledons (Figure 1A–C). In the analyses of lignified sites of ectopic lignin deposition in the roots of *lig* mutants, the macro was also used to select and measure only newly lignified areas in root tips of *lig* mutant (Figure 1D–F).

The time course of changes in lignified areas and *a** and *L** indexes in cotyledons of L*er* and Col are shown in Figure 2. In L*er*, new lignin deposition started one day after wounding, and the lignified area significantly increased for up to three days. The *a** and *L** indexes also significantly increased for up to three days. In contrast, new lignin deposition in Col started at one day after wounding and lignified area and *a** and *L** indexes showed a tendency to increase for up to two days, however, the difference was not statistically significant. Three days after wounding, lignified area coverage and *a** and *L** indexes of L*er* were about ten-fold higher than those observed in Col.

The time course of changes in the lignified area and *a** and *L** indexes of ectopic lignin deposition in the roots of *lig* mutants is shown in Figure 3. During cultivation at 28°C, definite lignin deposition was detected after two days of cultivation, and the lignified area and *a** and *L** indexes significantly increased for up to three days of cultivation in *lig* mutants. These results suggest that our analysis method is efficient for assessing the degree of stress-induced lignin deposition in cotyledons and roots of *A. thaliana*.

LigninJ facilitated collection of sufficient data to perform non-parametric tests. Regarding L*er* (Figure 2A–C) and *lig* (Figure 3), lignified areas and *a** and *L** indexes showed a steady increase in a time-dependent manner. This indicates that the measurements of lignified area and *a** and *L** indexes acquired through this macro program are objective and can be used to evaluate the degree of stress-induced lignin deposition. Moreover, lignified area measurements are accessible and useful to assess stress-induced lignin deposition.

In the CIELAB color space, the *L** component indicates the lightness value, and the *a** component indicates the red value. Although the *a** value is not consistently correlated with the intensity of the Wiesner reaction, the $100-L^*$ value is correlated with the intensity of staining. Therefore, to compare stress-induced lignin deposition in a defined area, the *L** index may be more useful than the actual lignified area and the *a** index.

For practical use, the macro program facilitates fast analysis of many samples and efficient testing of differences between groups. The macro can also to define and save the entire analytical process. Considering these advantages, our macro program may be useful for studying stress-induced lignification. Furthermore, the macro can also be used for other biological analyses based on different staining techniques or color regions in organisms. In conclusion, LigninJ offers considerable benefits for analyzing various kinds of images in biosciences.

Acknowledgements

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