

Quantification of stimulus-induced callose spots on plant materials

Masaki Kohari, Kohei Yashima, Yoshitake Desaki*, Naoto Shibuya*

Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama-ku, Kawasaki, Kanagawa 214-8571 Japan

*E-mail: desaki@meiji.ac.jp; shibuya@meiji.ac.jp Tel & Fax: +81-44-934-7039

Received September 9, 2015; accepted November 20, 2015 (Edited by K. Suzuki)

Abstract Plants are known to synthesize and accumulate callose, a β -1,3-linked glucan, in response to pathogen infection, elicitor treatment and wounding. Although callose deposition has often been used as an index of defense responses in plant, its quantification is laborious and difficult. We have developed a simple computer program to quantify callose spots on microscopic images based on multiple Rotational Morphological Processing (RMP), which is suitable for the detection of biological spots with intricate morphology. The program developed in this study is useful for the detection of callose spots on various biological backgrounds, making it possible to compare the responses of plant materials with different genetic background or different stimuli. Validity of this program was supported by comparing the results obtained by the new method and conventional manual counting. This program can be applicable to wide range of images taken by various microscopic systems and also in variable formats.

Key words: Aniline blue, callose, chitin, computer program, flagellin, MAMP, spot counting.

Plants are known to synthesize and accumulate callose, a β -1,3-linked glucan, in response to pathogen infection, elicitor treatment and wounding (Chen and Kim 2009; Voigt 2014). Well known example is the accumulation of callose as a major component of papillae, which is an apposition of a specialized cell wall at the site of attempted penetration of fungal or oomycete pathogens and plays an important role as the first line of defense (Collinge 2009; Underwood and Somerville 2008; Voigt 2014). Hence, the accumulation of callose has been used as an index of defense responses induced by pathogen infection and elicitor treatment. Callose accumulation has been analyzed by fluorescence microscopic observation of callose deposits stained by aniline blue on the leaves/tissues (Ellinger et al. 2013; Oliveira-Garcia and Deising 2013). Although fluorescence spectroscopic determination of solubilized callose using aniline blue was also reported for the analysis of callose synthase activity in membrane preparations (Shedletzky et al. 1997), we found it is difficult to apply the spectroscopic method for plant materials because of the high background of the alkaline extract of plant leaves.

Although quantification of callose deposits has often been done by manual counting of the callose spots within the limited area of leaves/tissues (Ali et al. 2013; Bohlenius et al. 2010), it is time consuming and sometimes biased by the judgement of individuals

engaged for counting. Quantification by conventional computer softwares such as ImageJ (Collins 2007; Ellinger et al. 2013) or Photoshop software (Luna et al. 2011) has also been utilized. Zhou et al., however, pointed out the difficulty to distinguish callose spots from complicated background images by these conventional softwares and developed a program, CalloseMeasurer, to quantify callose deposition (Zhou et al. 2012). While this program itself is an excellent one, it can only be applicable for a microscope equipped with a special software (Acapella, PerkinElmer, Inc.), limiting the general usage of this program.

We here report a simple program for automatic counting of callose spots, developed based on Rotational Morphological Processing (RMP), which is suitable for the detection of biological spots with intricate morphology (Kimori et al. 2010). This new program can be applicable to wide range of images taken by various microscopic systems and also in variable formats. It can detect callose spots on various biological background and quantify them, thus making it feasible to compare the responses of various plant materials with different genetic background and different stimuli.

Abbreviations: RMP, rotational morphological processing; MAMP, microbe-associated molecular pattern; SE, structuring elements.

This article can be found at <http://www.jspcmb.jp/>

Published online January 21, 2016

Materials and methods

MAMPs and plant materials

N-Acetylchitoheptaose was prepared by re-*N*-acetylation of chitoheptaose (chitosan heptasaccharide), kindly supplied by Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan). Flg22 peptide was synthesized by Peptide Institute, Inc. (Osaka, Japan). The *cerk1-2* (096F09), obtained from GABI-Kat, was described previously (Miya et al. 2007). KO mutant *fls2* was supplied by Dr. Ken Shirasu of RIKEN and *pmr4-1* (CS3858) was obtained from ABRC.

MAMP treatment and fluorescence microscopic observation

Sterilized *Arabidopsis* seeds were germinated on 24-well plates containing liquid MS medium with 1% sucrose. Seedlings were grown for 9 days under 16/8-h light/dark cycle at 22°C. Ten micromolars of *N*-acetylchitoheptaose or 100 nM of flg22 was added to the germinated 24-well plates, and incubated 18 h under constant light on the rotary shaker. After the incubation, the seedlings were immobilized with a 3:1 ethanol:acetic acid solution, rehydrated and stained with aniline blue as described previously (Shinya et al. 2014). Fluorescence of stained callose

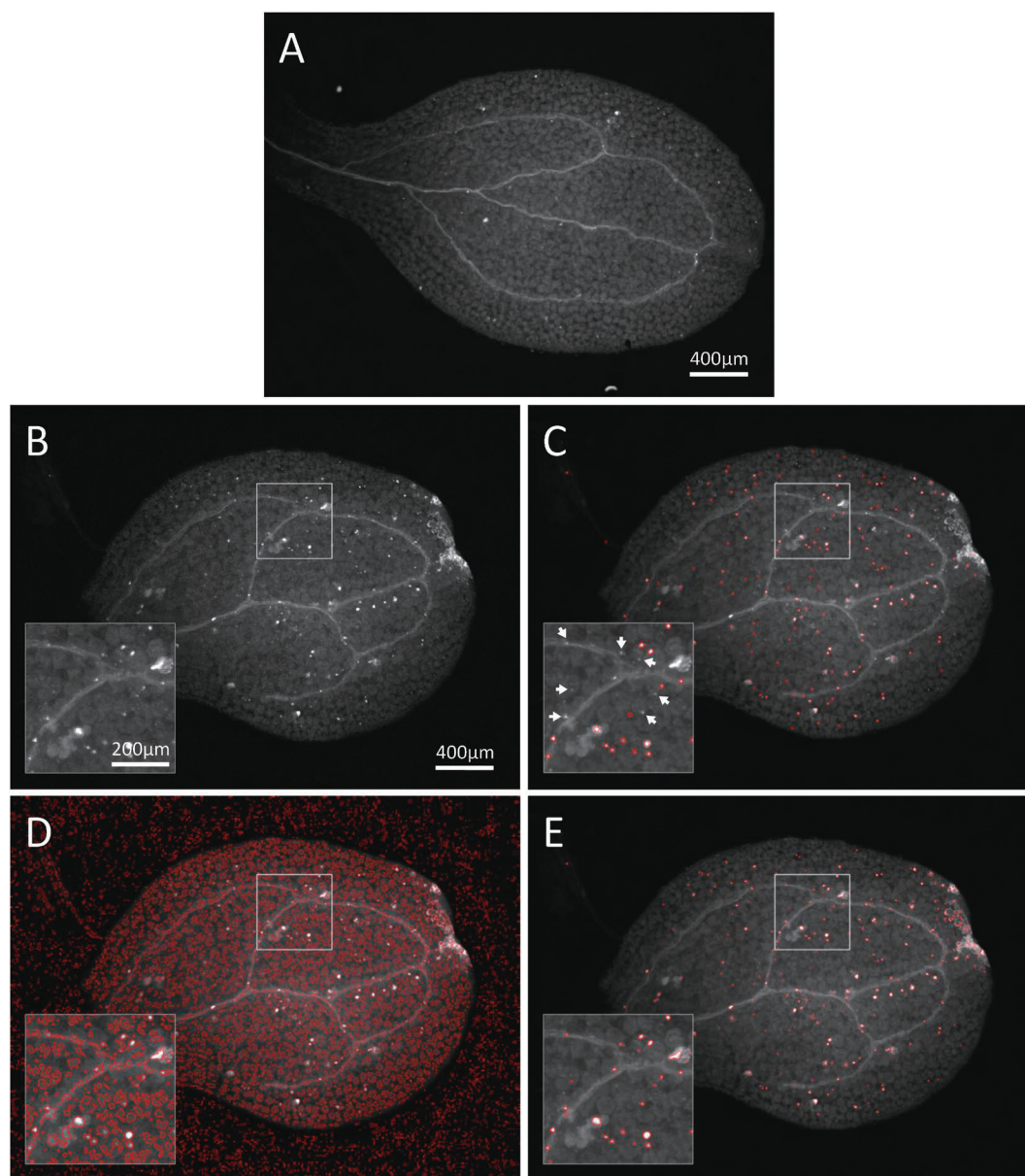


Figure 1. RMP analysis of chitin-triggered callose deposition on *Arabidopsis* leaves. (A) Fluorescence microscopic image of the control leaf treated with water for 18 h and stained with aniline blue. (B) Fluorescence microscopic image of the leaf treated with 10 μ M (GlcNAc)₇ for 18 h. Inset shows the enlarged image of the corresponding part of the original image. (C) Detection of the callose spots by single RMP (Upper limit, 7 pixel; lower limit, 5 pixel). Detected spots were outlined in red. Undetected callose spots were indicated by arrows. (D) Detection of the callose spots with a modified setting of upper/lower limits (Upper limit, 19 pixel; lower limit, 3 pixel). Note that numerous background objects were also detected (odd shaped objects outlined in red). (E) Setting a proper “threshold” parameter to the conditions in (D) improved selective detection of the callose spots.

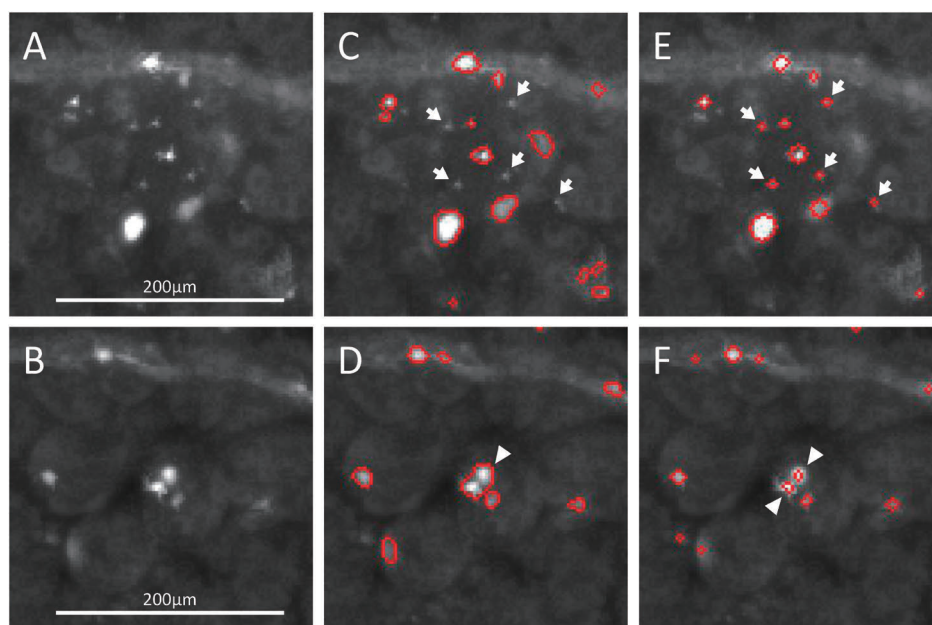


Figure 2. Comparison of single and multiple RMP for the detection of callose spots. (A) and (B) Magnified images of different parts of Figure 1 (B). (C) and (D) Callose spots in the area of (A) and (B) were detected by the single RMP with proper setting of upper/lower limit and threshold as described in Figure 1 (E). Note that the presence of small, undetected callose spots (arrows) and a merged spot (arrowhead). (E) and (F) Improvement of spot detection by the multiple RMP. Those spots not detected by the single RMP in (C) and (D) were clearly detected as separate spots (arrows and arrowheads).

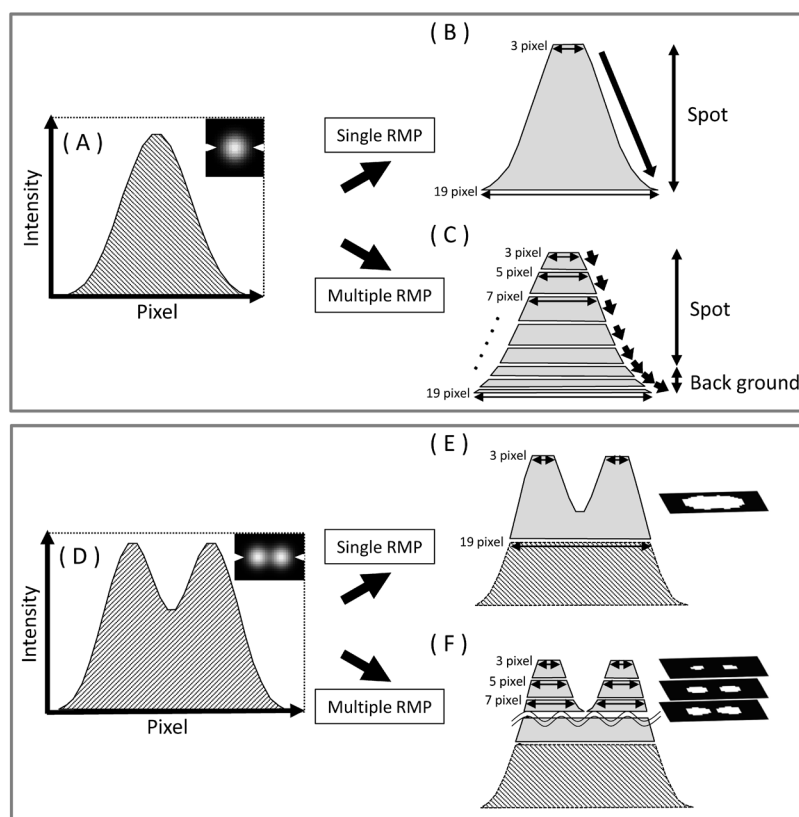


Figure 3. Basic concept of multiple RMP. (A) and (D) Model images (insets) and their intensity profiles. (B) Detection of intensity gradient of the image (A) by single RMP. (C) Detection of intensity gradient of the image (A) by multiple RMP. Note that the multiple RMP can detect the changes in intensity gradient more sensitively. (E) and (F) Detection of the closely associated spots in the image (D) by single and multiple RMP, respectively. In the case of the multiple RMP, two closely associated spots can be detected separately in the stepwise RMPs with the smaller sets of SEs, whereas the single RMP only detects these spots as a large, merged spot.

deposits was observed using BIPREVO BZ-9000 fluorescence microscope (KEYENCE, <http://www.keyence.com/>) at 390 nm excitation and 460 nm emission. Six images with different focus depth were combined to generate a fully focused image. Callose spots on the cotyledon was counted manually or by the new program reported here. In both cases, callose spots were counted for a defined area of each cotyledon. Usually 6 cotyledons were used to evaluate a mutant or treatment and the 4 results, except the maximum and minimum values, were used to calculate the average.

Spot detection by the single/multiple RMP

The single/multiple RMP program developed in this work was set up as a plugin file for the Icy, which is a software provided by the Quantitative Image Analysis Unit at Institut Pasteur (<http://www.bioimageanalysis.org/>). Region of images to be analyzed was determined by the icy original tools. To detect callose spots using single RMP, fluorescence images were first smoothed with Gaussian filter (3×3 kernel). Spots were then extracted by an appropriate set of structuring elements (SE), of which sizes were selected manually based on the sizes of minimum and maximum callose spots (e.g., from 3 to 19 pixel). Binarization of the image was performed using an appropriate threshold. The noises were removed by the conventional morphological opening using disk SE, of which size was determined by the smaller segments. In the case of multiple RMP, spot extraction was repeated for every two pixels from minimum to maximum diameter of callose spots. After binarized and denoised, each image was combined to make a merged image.

Results

Detection of callose spots by single RMP

We applied RMP to detect and quantify the callose spots on plant leaves induced by MAMP treatment and stained with aniline blue. RMP is a mathematical method developed for the efficient extraction and characterization of biological spots in various types of images such as fluorescence/electron microscopic images (Kimori et al. 2010). RMP obtains the information by rotating the original image with respect to a narrow (1 pixel) segment (SE) with a distinct length and incorporating the intensity of black and white color, fluorescence etc. within the area. In the original RMP method, a set of SE with the size of arbitrary defined upper and lower limits, are used as a moving probe along each pixel to cover whole range of the image. Accumulated information for all the SEs are integrated to extract biological spots in the image. RMP can limit the size of the objects to be analyzed by setting the upper and lower limit of SE. This method can also eliminate noises such as small dots or extremely elongated objects by smoothing treatment with Gaussian filter and cleaning with the lower limit SE (Kimori et al. 2010).

Application of RMP for the detection of callose spots on the Arabidopsis leaves treated with a chitin oligosaccharide, (GlcNAc)₇, could extract the callose spots formed on the leaves (Figure 1B and C, Figure 1A for water control). However, depending on the setting of SE, some spots larger or smaller than the upper/lower limits were eliminated (Figure 1C, arrows). When the upper/lower limits were set to include most of the callose spots, coverage of the spots was significantly improved (Figure 1D). At the same time, however, numerous background images were also detected as callose spots with this setting (Figure 1D, odd shaped images outlined in red). To eliminate such noises, we introduced a criteria of “threshold”, which distinguishes callose spots from these backgrounds based on the differences in their fluorescence intensity. Proper setting of threshold eliminated most of the background images and dramatically improved the extraction of callose spots (Figure 1E).

Improvement of spot detection by multiple RMP

Although we could detect callose spots on the

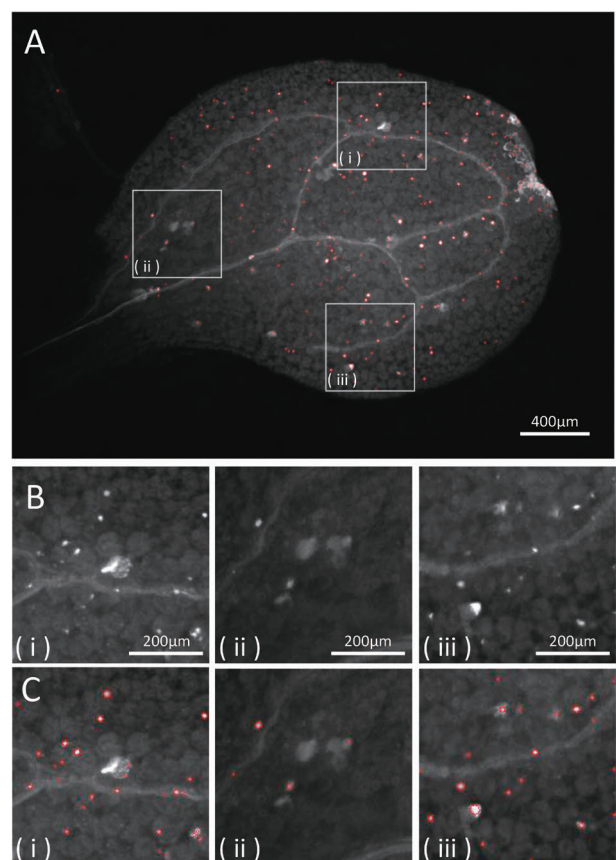


Figure 4. Application of the multiple RMP enabled the selective detection of callose spots on the leaves with diverse background objects. (A) Detection of callose spots by the multiple RMP. (B) and (C) Magnified images of the area (i)–(iii) in (A), which contained leaf veins, irregular shaped background objects and blurs. (B), fluorescence microscopic image; (C), detection of callose spots by the multiple RMP.

(GlcNAc)₇-treated leaves as described, detailed analysis of the results revealed that some small spots were still eliminated (Figure 2A and C, arrows) and closely associated spots were detected as a merged spot (Figure 2B and D, arrow head). It was suggested that such problems mostly originated from the setting of upper/lower limit of SE, which covers all the sizes of the callose spots, and also the significantly high value of threshold, which was forced to use to eliminate background noises. To elucidate these problems, we introduced multiple, stepwise RMP by successively changing the size of SE for each RMP (Figure 3). By introducing the multiple RMP, which makes possible to reconstitute the intensity gradient of each image from a set of incorporated data, it was expected to distinguish callose spots and background noises more precisely based on the differences in the intensity gradient of these images (Figure 3A–C). It was also expected that the multiple RMP could distinguish closely associated spots as separate ones based on the

changing sets of SEs (Figure 3D–F). In fact, application of such multiple RMP with proper setting of threshold enabled not only to distinguish callose spots from the background but also to detect some callose spots, which could not be detected or separated by a single round RMP, accurately (Figure 2E and F, arrows and arrowhead). This improved multiple RMP method could distinguish callose spots from various biological backgrounds, including leaf veins, irregular shaped objects and blur, on micrographic images (Figure 4A–C).

Validation and application of the multiple RMP for callose spot counting

To evaluate the validity of the multiple RMP for callose spot counting, we compared the results of callose spot detection by the multiple RMP and manual counting (Figure 5). As shown, both methods showed a very similar results for the estimation of callose deposits on MAMP-treated leaves of Col-0 and several mutants for

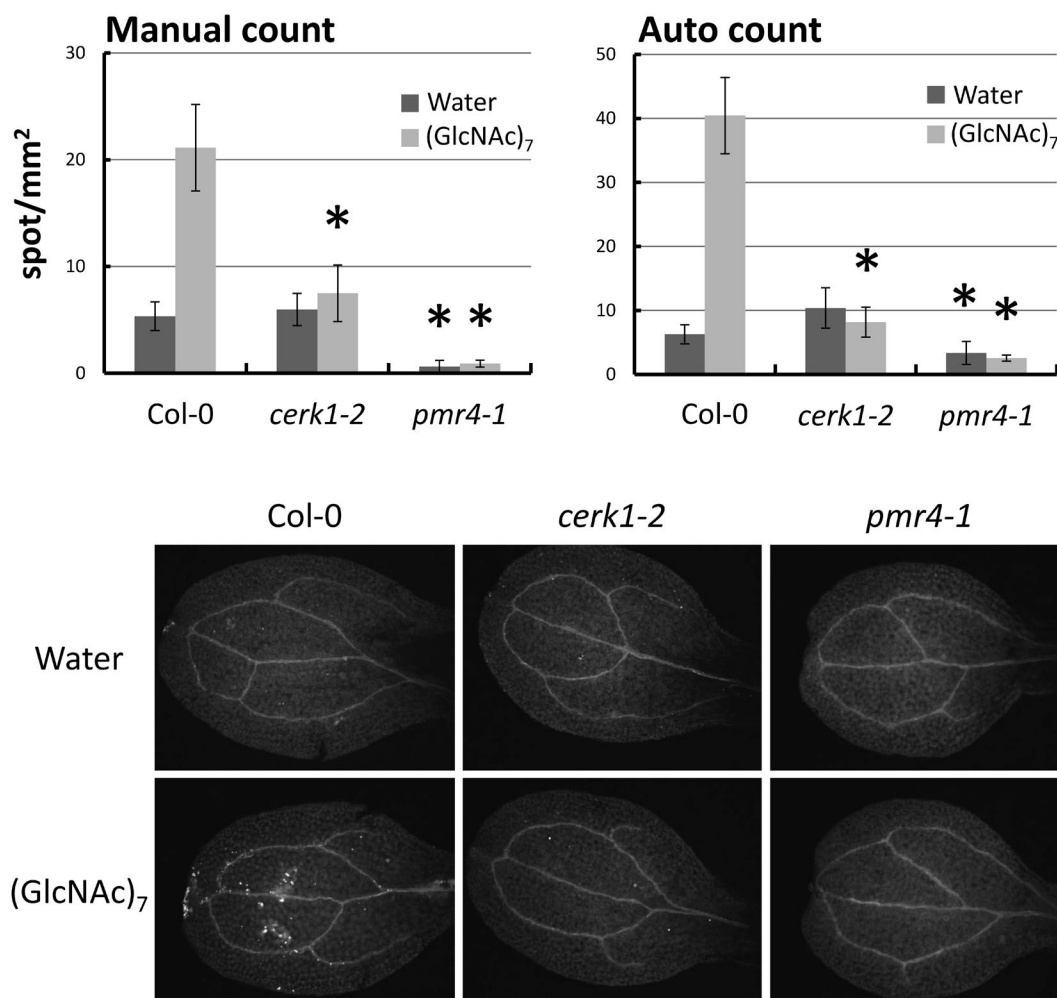


Figure 5. Comparison of manual and auto counting of callose spots. Callose spots on the leaves treated with (GlcNAc)₇ were counted manually (upper left) or by the auto count program described here (upper right). Error bars indicate standard deviation. The asterisks indicate statistical significance (Student's *t*-test, $p < 0.05$) between the same treatments of wild type Col-0 and the corresponding mutants. Representative images for these treatments were shown at the lower part of the figure.

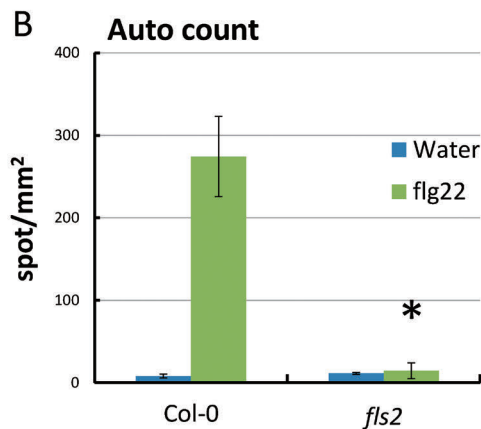
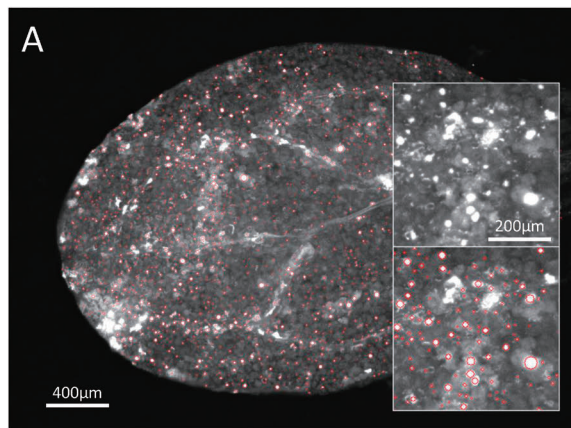


Figure 6. Detection of the callose spots induced by flg22 treatment. (A) Detection of callose spots induced by flg22 on an Arabidopsis leaf. Arabidopsis seedlings were treated with 100 nM flg22 for 18 h before microscopic observation. (B) Quantification of the callose spots by the auto count program. Error bars indicate standard deviation. The asterisk indicates statistical significance (Student's *t*-test, $p < 0.05$) between the same treatments of wild type Col-0 and *fls2*.

MAMP signaling. Mutant of CERK1 (*cerk1-2*), which is essential for chitin signaling (Miya et al. 2007), did not accumulate callose deposits by (GlcNAc)₇ treatment, confirming the specificity of chitin response. Similarly, *pmr4-1* mutant lacking the callose synthase responsible for pathogen/MAMP-induced callose accumulation (Jacobs et al. 2003; Nishimura et al. 2003) did not show (GlcNAc)₇-induced callose deposits. The difference between the *cerk1-2* and *pmr4-1* mutants indicated that PMR4 contributes to the basal callose accumulation in the non-stimulated plants.

To further verify the validity of this method, we analyzed the callose accumulation by a different MAMP molecule, flg22, which is known to induce callose accumulation in Arabidopsis (Gomez-Gomez et al. 1999). As shown in Figure 6, flg22 induced callose deposition more intensely than chitin oligosaccharides and the deposits were successfully counted by the present method. This callose deposition was observed in Col-0 but not in *fls2*, which lacks flg22 receptor. This method

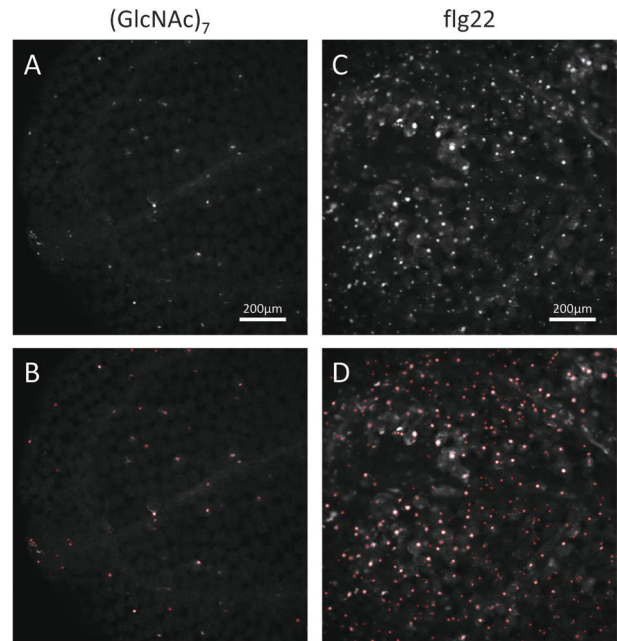


Figure 7. Application of the multiple RMP to laser confocal microscopic images. (A) and (C) Laser confocal microscopic images of callose spots induced by (GlcNAc)₇ or flg22 treatment. (B) and (D) Detection of callose spots by the multiple RMP.

could also be applied to the microscopic images obtained by confocal laser scanning microscopy (Figure 7), indicating the flexibility for different types of images.

Discussion

A simple program to quantify callose deposits on plant materials was developed based on the multiple RMP and background elimination by setting appropriate threshold for the gradient of fluorescence intensity. Despite the simple logics adopted by this program, it was shown to be useful for the quantification and comparison of callose deposits between different stimuli or different mutants. The simplicity of the program enabled it to be operated on conventional computer systems available in most laboratories. This program as well as the operation manual can be downloaded freely from <http://bioinf.mind.meiji.ac.jp/Callose/>.

This method has several advantages, such as time-saving and elimination of errors originating from differences between individual judgements, compared to the conventional manual counting. Correlation between the results obtained by manual and automatic counting was generally good. Although the automatic counting detected more callose spots as shown in Figure 5, it seems difficult to conclude which result reflects real situation better.

This program can be applicable to wide range of images obtained by using various types of microscopes as shown for the images taken by conventional fluorescence

microscopy and confocal laser scanning microscopy. As the “opening and closing” treatment in this program, which was designed to remove noises, regenerates a shape that is not necessarily the same to the original shape, estimation of the area of a spot that differs from circular shape could contain some error. For this reason and also the differences in the focus of the callose spots in conventional microscopic images, we recommend to use this program to count callose spots rather than to calculate the area of callose spots.

Acknowledgements

We thank Dr. Hanae Kaku of Meiji University for useful advices. We also thank Dr. Kentaro Yano and Mr. Koji Yokoyama of Meiji University for the help in constructing the web site for the software distribution. We are indebted to Yaizu Suisankagaku Industry Co., Ltd. for the supply of chitosan oligosaccharides. This work was supported in part by Grants-in-Aid for Scientific Research (No. 26650104 to N.S.), Grants-in-Aid for Young Scientists (No. 26850028 to Y.D.) and Grants-in-Aid for Scientific Research on Innovative Areas (No. 25114516 to N.S.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Ali MA, Abbas A, Kreil DP, Bohlmann H (2013) Overexpression of the transcription factor RAP2.6 leads to enhanced callose deposition in syncytia and enhanced resistance against the beet cyst nematode *Heterodera schachtii* in Arabidopsis roots. *BMC Plant Biol* 13: 47
- Bohlenius H, Morch SM, Godfrey D, Nielsen ME, Thordal-Christensen H (2010) The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley. *Plant Cell* 22: 3831–3844
- Chen XY, Kim JY (2009) Callose synthesis in higher plants. *Plant Signal Behav* 4: 489–492
- Collinge DB (2009) Cell wall appositions: the first line of defence. *J Exp Bot* 60: 351–352
- Collins TJ (2007) ImageJ for microscopy. *Biotechniques* 43(Suppl): 25–30
- Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA (2013) Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. *Plant Physiol* 161: 1433–1444
- Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J* 18: 277–284
- Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* 15: 2503–2513
- Kimori Y, Baba N, Morone N (2010) Extended morphological processing: a practical method for automatic spot detection of biological markers from microscopic images. *BMC Bioinformatics* 11: 373
- Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: A multifaceted plant defense response. *Mol Plant Microbe Interact* 24: 183–193
- Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc Natl Acad Sci USA* 104: 19613–19618
- Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301: 969–972
- Oliveira-Garcia E, Deising HB (2013) Infection structure-specific expression of β -1,3-glucan synthase is essential for pathogenicity of *Colletotrichum graminicola* and evasion of β -glucan-triggered immunity in maize. *Plant Cell* 25: 2356–2378
- Shedletzky E, Unger C, Delmer DP (1997) A microtiter-based fluorescence assay for (1,3)- β -glucan synthases. *Anal Biochem* 249: 88–93
- Shinya T, Yamaguchi K, Desaki Y, Yamada K, Narisawa T, Kobayashi Y, Maeda K, Suzuki M, Tanimoto T, Takeda J, et al. (2014) Selective regulation of the chitin-induced defense response by the Arabidopsis receptor-like cytoplasmic kinase PBL27. *Plant J* 79: 56–66
- Underwood W, Somerville SC (2008) Focal accumulation of defences at sites of fungal pathogen attack. *J Exp Bot* 59: 3501–3508
- Voigt CA (2014) Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Front Plant Sci* 5: 168
- Zhou J, Spallek T, Faulkner C, Robatzek S (2012) CalloseMeasurer: A novel software solution to measure callose deposition and recognise spreading callose patterns. *Plant Methods* 8: 49