Original research article

RootLM: a simple color image analysis program for length measurement of primary roots in *Arabidopsis*

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Abstract: The *Arabidopsis* root is a model system for studying root growth, development, and response to environmental stresses. Due to its small size, many *Arabidopsis* seedlings can be cultured in a single nutrient-containing agar plate. Two of the common parameters used to evaluate root growth are the root length and the elongation rate. Various methods including manual measurement and digital image analysis can be used to measure the root length. In this paper, we report the development of a simple color image analysis program, RootLM, in combination with a modified marking technique of root growth on the surface of a Petri dish, for length measurement of primary roots. Our method provides an alternative tool to quickly generate accurate and reproducible root growth measurement with a minimum equipment requirement. In addition, the program is free for public download.

Keywords: *Arabidopsis*, elongation, image analysis, roots, RootLM

Introduction

Roots are a hidden half of plants and are essential for plant life. Their roles include water and nutrient uptake, anchorage for shoots, synthesizing and supplying active bio-molecules, sensing stress, and storage. Due to its residence in soil, root growth and development are less studied than leaves and stems.

Arabidopsis thaliana is a model system for plant research (Meinke et al. 1998). Due to its small size, many *Arabidopsis* seedlings can be cultured on an agar or Phytagel medium in a Petri dish. Since roots are readily visible on the surface of the transparent medium, this culture system is excellent for analysis of root growth and development (Scheres et al. 2002). For instance, identification and characterization of mutants in *Arabidopsis* have greatly enhanced our knowledge in root development and root hair formation (Schiefelbein and Somerville 1990, Benfey et al. 1993, Meinke et al. 1998).

Since root growth and development are very sensitive to many environmental stresses and growth factors, root growth has been used as an indicator for screening mutants that have altered responses to stresses or growth hormones. For example, salt-over-sensitive (SOS) mutants with a severe reduction in root elongation were identified from a mutant *Arabidopsis* population grown on a NaCl-containing agar medium in a Petri dish (Ishitani et al. 1998). Further studies of these mutants have led to the discovery of the SOS-salt response signaling pathway (Zhu 2002).

In the process of studying the laccase gene family (17 annotated genes) in *Arabidopsis*, we found that the transcript level of many laccase genes in the family was up-regulated by salinity and PEG-induced dehydration treatments in primary roots. To provide genetic evidence for the roles of these laccase genes in stress responses, we identified mutants for most genes in the family and examined primary root elongation of these mutants in comparison with wild-type plants, when grown on a Phytagel medium containing different concentrations of NaCl or on PEG-treated plates (dehydration stress) using the Petri dish system (Cai et al. 2006). To minimize changes in growth conditions during an experiment, root growth was usually tracked by tracing roots on the surface of a Petri dish and all the traces were measured manually afterward. However, manually collecting root elongation and root length data turned out to be challenging. For a plate with an average of 16-20 roots that were marked daily, it usually meant that more than 100 data points needed to be collected and entered the computer. This was a very time-consuming, tedious, and error-prone process. For a large-scale experiment involving 10 to 20 plates, it also was quite tiresome.

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Various computational methods were developed in the past to facilitate root length measurement (Newman 1966). A combination of video photographic methods or scanners with computers further improved the process of root length measurement (Voorhees et al. 1980, Pan and Bolton 1991, Kaspar and Ewing 1997). Some of the software such as commercially available RHIZO (Regent Instruments, Quebec, Canada) are sophisticated and can measure root diameter in addition to root length. For our *Arabidopsis* research project, a relatively quick and simple method for measuring the primary root length will be sufficient. To this end, an image analysis system (NIH-Image, written by Wayne Rasband at the NIH, USA) was employed by plant biologists to measure root growth in *Arabidopsis* (Doerner 2002). In this system, the markings of root growth at different time points on plates are acquired as images and are retraced on a computer for further analysis of the root length. However, it normally requires a steady hand to retrace markings on a computer in order to accurately measure the root length. In this study, we modified the marking technique by using blue and red color markers alternately to trace the daily primary root growth on plates. The color image was then acquired and analyzed using a simple Matlab program, RootLM, to directly measure lengths of individual markings or daily growth and to compute the total root lengths. The root elongation rate can be calculated if the time interval is known for each marking.

Materials and Methods

Plant Culture and Root Growth Marking

Twenty seeds of *Arabidopsis thaliana* (Columbia 0 ecotype) were surface sterilized and arranged on the surface of a culture medium in a square Petri dish (10 x 10 x 1.5 cm^3). The solid medium consisted of 0.5X MS salt, 0.5% sucrose, 10 mM MES, and 0.6% Phytagel (Sigma, St. Louis, MO) at pH 5.8. The Petri dishes with seeds were wrapped with two layers of Saran wrapper strips (2 inch wide). After a cold treatment (4°C) for two days in the dark, the seeds were germinated and seedlings were grown on a light shelf under a 16/8 h light/dark photoperiod at room temperature. Light was supplied by four cool-white florescent bulbs, reaching an intensity of approximately 120 µmol m^{-2} s^{-1} on the shelf surface. The plates were placed vertically on a rack so that the roots grew downward on the medium surface. Root growth was tracked daily by marking new growth of individual roots on the back surface of the Petri dish (Wu et al. 1996). In order to use our developed software for calculating the root length, blue and red colored-Sharpie Twin-tip (fine/ultrafine) permanent markers (Sanford Corporation, Oak Brook, IL) were used alternately for marking. To speed up the marking process and minimize changes in growth conditions during markings, the ultrafine tips of the markers were used and the plates were returned to the original growth environment immediately after each marking. Since one root would occasionally cross over another root, a berry-colored marker pen was used at the intersection.

Once the primary roots reached the bottom of the vertically placed Petri dish or at the end of each experiment, the Phytagel growth medium and plants were removed, and the Petri dish with markings was rinsed and blotted dry carefully with Kimwipes (Kimberly-Clark Inc., Ontario, Canada). The markings were retraced with the fine tips of the same color to ensure that each root replica is a connected solid line of two-color segments. The plates were then arranged on a flat-bed scanner (HP Scanjet 3570C, WIA-EPSON Perfection 1650 or other scanners) and scanned at 300 dpi. The scanned images could be viewed and preprocessed by any image editing software such as Adobe Photoshop Elements (Adobe Systems Incorporated, San Jose, CA). Specifically, the image of each plate was cropped and saved as an individual new "JPEG" image file with a size of 945 by 945 or any appropriate lower or higher resolution. Some representative images are shown in Figure 1. Before feeding the plate image into our software, each image was visually examined to ensure each root replica was a connected solid line of two-color segments without contact with adjacent root replicas. A "paint brush" tool with a white color was used occasionally to cover color smearing caused by marking and to separate two roots that are too close to each other (Figs. 1b and 1c).

Root Length Measurement

To calculate the primary root length, the image was analyzed using the RootLM program (see details below). The RootLM program is not a stand-alone program. Currently, it runs under Matlab Version 7.0.1 and Image Processing Toolbox Version 5.0.1 (The Mathworks, Inc., MA). The program provides a user friendly graphical interface for loading one or multiple images (supplemental Fig. 1). The user can select the "Measure single" or "Measure All" button to process a single or multiple plate images. The program will process the loaded image(s), display the intermediate results, and save the final measurement in a data file. This data file lists the lengths of each segment (such as daily marking or daily growth) for all the roots on the plate and summarizes the total length of each root. The data can also be easily copied and pasted into the Microsoft Excel program (Mi-

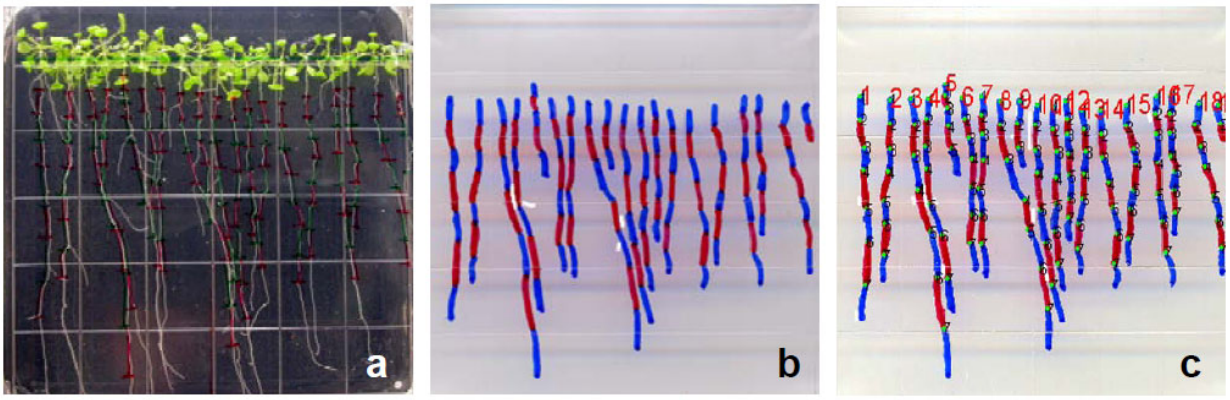

Fig. 1. Representative images of *Arabidopsis* seedlings and root markings. (a) An image of cultured *Arabidopsis* seedlings in a Petri dish with initial markings for daily growth of roots. (b) A scanned image from the retraced root markings on the same Petri dish. (c) A representative image after seven processing steps using RootLM. Some white paint brush streaks were used to cover some color dots or marking smears and were visible in (b) and (c).

crosoft Cooperation, Seattle, WA) for calculating the elongation rate.

The step-by-step algorithm of the RootLM is summarized as follows:

Step 1: Generate a binary segmented root image by applying an empirically determined threshold value in the saturation subspace of the HSV (Hue, Saturation, and Value) color space.

Step 2: Remove noise in the segmented image by a series of morphological operations (supplemental Fig. 2a).

Step 3: Convert the color image to a grayscale image by masking the binary segmented root image with the original image and setting red, blue, and berry components as an intensity of 50, 150, and 250, respectively (supplemental Fig. 2b).

Step 4: Generate a skeleton image, which contains all the roots with the width of each root being 1 pixel, using the object thinning algorithm (supplemental Fig. 2c).

Step 5: Determine each root segment using the connected component method. The broken root segment due to the intersection of two roots, as indicated by the berry color, will be reconnected based on the trend of the broken line segment (supplemental Fig. 2d).

Step 6: Compute the length of each segment using a variant of the Euclidean distance, where the number of pixels along the root segment is divided by the cosine angle between the straight line connecting the two end points of the root segment and a vertical line. This operation makes a reasonable adjustment on the final root length when the primary root is not straight.

Step 7: Convert the length in pixel to the length in millimeter based on the aspect ratio of the input image. The outputs are the length of individual segment (daily root growth) and the total length of each root.

For a detailed root measurement protocol using RootLM and for downloading the RootLM software, please visit the web site: http://www.cs.usu.edu/~xqi/RootLM/.

Results and Discussion

A typical Petri dish with cultured plants is shown in Figure 1. Initial markings made with ultrafine tips are visible on the lid of the Petri dish (Fig. 1a). The markings were retraced with fine tips and then scanned using a flat bed scanner (Fig. 1b). The saved image file was finally processed by the RootLM program. A typical processed image is shown in Figure 1c. The length of each root segment was automatically saved in an ASCII text data file, which could be easily copied and pasted into a spreadsheet. The data file lists the length of each segment (daily marking or growth) of individual root in a column and summarizes the total root length at the end of each column. Daily elongation rate could be easily calculated if the time interval is known for each marking.

One of the major concerns about this image analysis program is whether it can reliably measure the trace and the root length. An experiment was thus conducted to compare the results obtained manually and obtained from the RootLM program using the same Petri dish shown in Figure 1. Six persons participated in the experiment. In order to assess the variation caused by hardware, five scanners from different manufacturers were used for acquiring the image. As shown in Figure 2a, the variation of the total root length measured by RootLM using images from different scanners is almost negligible (0.11 mm on average), while the difference made among six people could reach 3~5 mm with an average of 1.3 mm (Fig. 2b). To ensure the same scale as used in Figure 2a, we plotted the manual measurement difference among four individuals in Figure 2b since the other two individuals made substantial mistakes in

entering data points. The variation of the individual segment (daily markings or growth) measurements caused by different scanners was also smaller when compared with the variation caused by different persons. Specifically, different scanners resulted in a variation within the range of ± 1 mm with an average of 0.13 mm (Fig. 2c), while different persons caused a variation within ± 2 mm with an average of 0.26 mm (Fig. 2d). It should be noted that the daily growth measurement obtained by our RootLM program has a slightly larger variation than the total root length measurement (Figs. 2a and 2c). This is mainly because the RootLM program detects the individual segments by recognizing the color trace of daily markings (alternating blue and red), whereas two segments with different colors overlap at the joint and slightly interfere with positioning the junction point (see green dots in Fig, 1c) of two segments. These overlapping color traces, therefore, caused a slight variation in lengths of two adjacent daily markings but had negligible effect on the sum of the two daily growths.

Since the overlapping of manually marked color traces affected the accuracy of the daily growth measurement, we wondered whether this variation was acceptable. To this end, we first averaged the total root length and the daily growth of each root measured by four persons who did not make mistakes in measuring and entering the individual daily growth, although variations in measurement existed among them. These averaged total root length and daily growth of each root were treated as the ground truth of the root measurement. The ground truth was then compared with the root measurement obtained by RootLM using the images from five scanners. Figure 3 shows the difference between the root measurement computed from each scanned image and the ground truth. It clearly demonstrates that the differences in both total root length (Fig. 3a) and daily growth (Fig. 3b) are small. In particular, the average differences for the total root length measurement and the daily growth measurement are 0.76 mm and 0.35 mm, respectively. Furthermore, these variations are usually smaller than the ones generated by the manual measurement based on the observations by comparing Fig. 3a with Fig. 2b and Fig. 3b with Fig. 2d. Root 7 in Fig. 3b seems to be an exception. However, a close examination of the measurement for root 7 revealed that the large variation in root 7 was mainly due to the differences among people during manual measurement (see Fig. 2d). Specifically, the measurements of two adjacent daily growths were different among persons, resulting in one root segment that was longer and the other was shorter than the measurement from RootLM. Another interesting observation is that the total root length of many roots from the color image analysis program was shorter than the ground truth

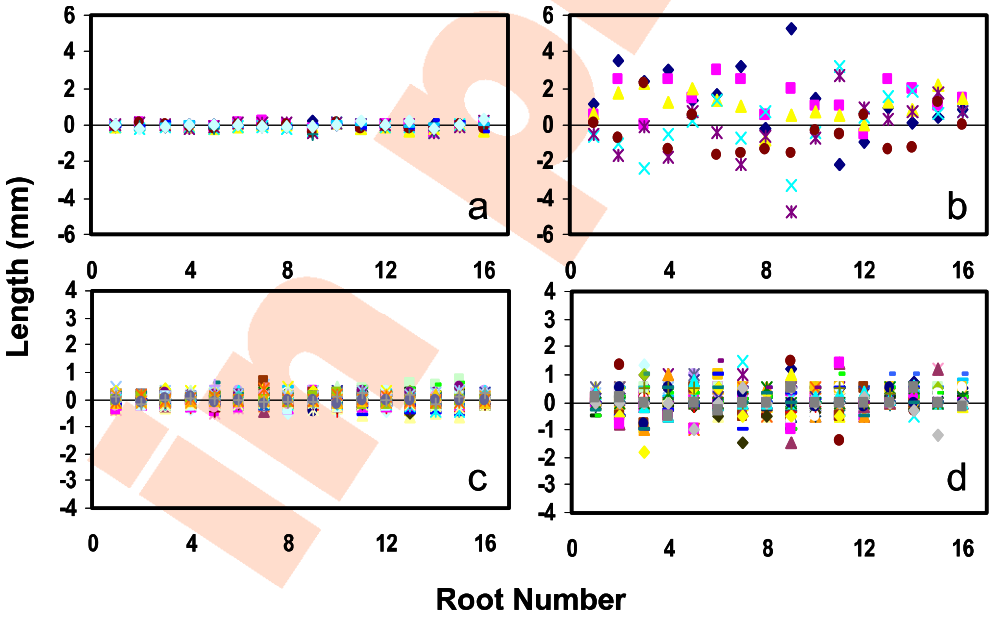

Fig. 2. Variations of root length measurement among different individuals (manual measurement) and from RootLM analysis using images from different scanners. (a) Variations of the total root length measured by RootLM using images acquired by five scanners. Each vertical cluster represents 10 data points from pairwise comparisons of five scanned images. Many symbols overlap due to the same or similar values. (b) Variations of the total root length measured by four persons. Each vertical cluster represents 6 data points from pairwise comparisons among four individuals. Some symbols overlap due to the same or similar values. (c) Variations of the daily marking measurement obtained by RootLM using images acquired by five scanners. Each root had seven daily markings and each daily marking was compared among the images obtained from five scanners, yielding 70 data points for each root. Many symbols overlap due to the same or similar values. (d) Variations of the daily marking measurement among four persons. Each root had seven daily markings and each daily marking was compared among four individuals, yielding 42 data points for each root. Many symbols overlap due to the same or similar values.

(Fig. 3a). One of the possible explanations for such a difference is a lower resolution of the manual measurement using a ruler. This low resolution makes it difficult to provide an accurate reading below 1 mm, and, therefore, the data from manual measurements are often rounded up. In addition, the junction point of two daily growths was usually measured twice when moving a ruler along the root during the manual measurement. However, the digital image analysis provides an accurate reading once each junction point has been determined.

We also made multiple measurements by applying RootLM on a plate image. The variation in both total root length and daily growth is zero, indicating a perfect reproducibility. However, multiple measurements of the same plate by the same person often produced some variations (data not shown). Thus, our results indicate that the measurement from the color marking and image analysis using RootLM is comparable to the manual measurement but with higher reproducibility.

Time savings is another advantage of using the image analysis program. While the time required for measuring a single plate was very similar between the manual and automated measurements, the time savings is significant when multiple plates are measured. In a comparison experiment, six plates were measured by six persons (6 plates/each person) with different levels of experience in manual root measurement or by RootLM operated by a trained user. After considering the additional time required for retracing markings on each plate prior to the use of RootLM, the color image analysis method yields up to 30~50% time savings compared with the manual measurement. This is because a total of six plates can be scanned in one run on a conventional flat bed scanner and multiple images can be sequentially processed in a batch by the RootLM program. Although retracing is still required in this new method, it occurs on plates instead of on a computer as employed in the NIH-image analysis program. Thus, the new method is much easier and more controllable. Currently, the RootLM program is implemented by Matlab 7.0.1. We are in the process of converting RootLM to a stand-alone program so that plant researchers can use it without purchasing the expensive Matlab software. In conclusion, the color marking and the image analysis method can provide quick, accurate, and reproducible measurement of the length of primary or unbranched roots and is an alternative tool for root growth measurement in *Arabidopsis*.

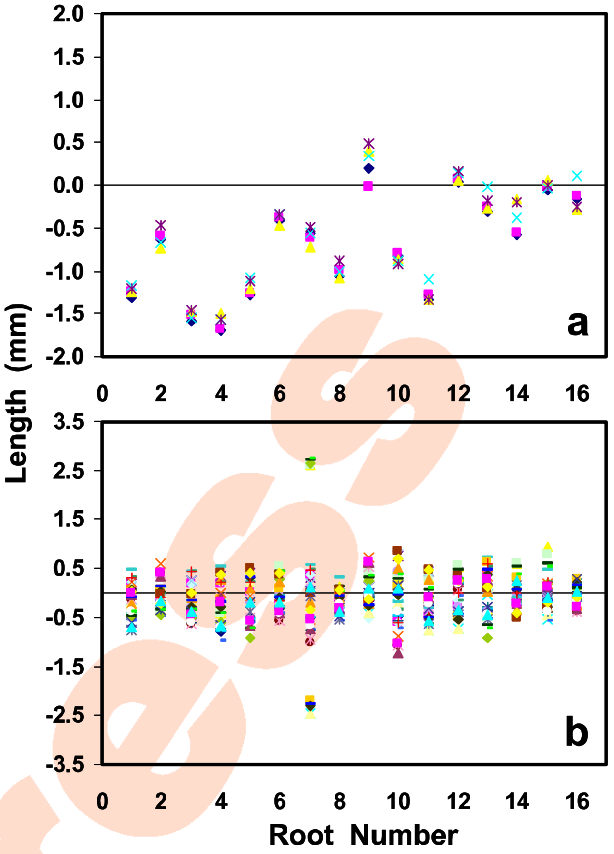

Fig. 3. Comparison of the ground truth of the root length measurement with the measurements obtained by the color image analysis method. Variations between the ground truth and the measurement from RootLM, using images acquired from five scanners, in total root length measurement (a) and daily marking (b).

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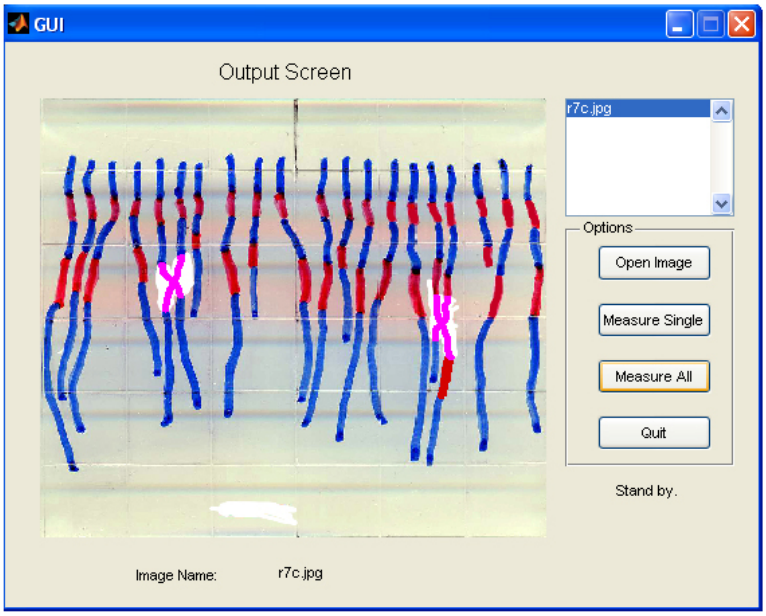
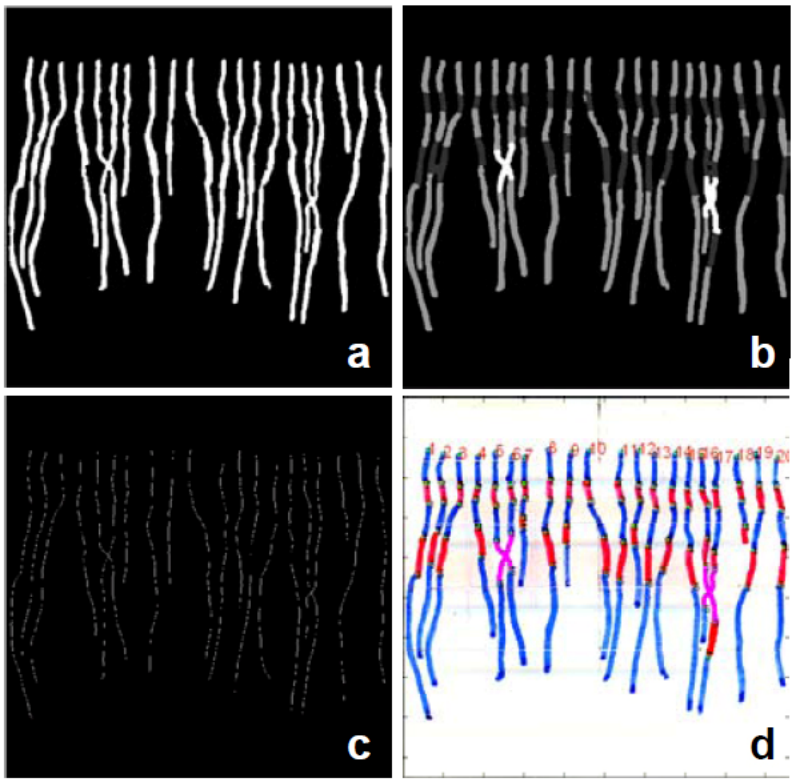
Supplemental Figures



Supplemental Figure 1. The graphical user interface of the RootLM software.



Supplemental Figure 2. The intermediate and final processing results of the RootLM software. The clean segmented root image (a); the grayscale root image labeled by three intensities (b); the skeleton root image with the width of each root being 1 pixel (c); final segmented roots with a green dot at each junction point (d). Note: Two sites of root intersection indicated by berry color were created artificially for testing the software.