Imageing of calcium gradient oscillations in plant root hairs by light sheet fluorescence microscopy

Giorgia Tortora¹, Stefano Buratti², Matteo Grenzi², Alex Costa², Andrea Bassi¹,* and Alessia Candeo¹

¹Politecnico di Milano, Dipartimento di Fisica, 20133 Milano, Italy
²Università degli Studi di Milano, Dipartimento di Bioscienze, 20133 Milan, Italy

Abstract. Root hairs are a delicate single-cell system whose growth is regulated by a fine mechanism characterised by the presence of a tip-high Ca²⁺ gradient that shows regular oscillations in growing root hairs. We show a method based on the use of Light sheet fluorescence microscopy (LSFM) which allows the quasi-physiological analysis of Arabidopsis thaliana plant roots hairs with excellent spatial and temporal resolution over a wide field of view. We show how the healthy growing root hairs are linked to precise oscillations and how a disruption of this mechanism can be associated to specific genes.

1 Introduction

Understanding the role of specific genes involved in the healthy growth of plants allows us to select for genes and traits which have a favourable impact on the development of more resilient plants. Specifically, tip growth controls the extension of both root hairs and pollen tubes. This happens thanks to a highly localised, tip-focused oscillating calcium gradient, maintained and regulated by specific calcium pumps, and channels. A direct link between healthy growth of root hairs and the oscillating behaviour of the calcium gradient has previously been reported [1]. To understand the involvement of specific genes in the correct growth of root hairs, genetically modified plants with the genes of interest knocked out can be created. By studying the variations in the oscillating behaviour of the calcium gradient, it is indeed possible to speculate on the physiological role of the knocked-out genes and in particular if they are active in the calcium dependent tip-growth of the root hairs. To visualise the calcium gradient, transgenic seedlings can be created expressing fluorescent genetically encoded calcium indicators. Standard microscopy has been used for the visualization of calcium dynamics with fluorescent probes in Arabidopsis thaliana, but with limitations due to the lack of sectioning capability for wide-field microscopy and to the high photodamage and small imaging volume for confocal and multiphoton microscopy. However, with standard microscopy methods the plants are usually placed horizontally on a slide. More particularly for the study of delicate single-cell structures like the root hairs, this arrangement can induce stress and the burst of the root hairs, making this type of study hard and prone to mistakes. As an alternative, we propose to use of light sheet fluorescence microscopy (LSFM) [2] and tailor a protocol around the plants requirements [3]. LSFM has become a fundamental resource for developmental biology in the last decade thanks to its capability of providing high resolution images of single cells over a wide field of view with high temporal resolution and low phototoxicity. In LSFM, a single plane of the sample is excited by a sheet of laser light, and its fluorescence is recorded in the orthogonal direction. The 90-degrees geometry is well suited to maintain seedlings vertically at almost physiological circumstances during the whole experiment. In this manner, spontaneous oscillation in root hairs of both wild type and knocked-out mutant plants were observed and analysed to understand the correlation between calcium regulation and the healthiness of growth.

2 Materials and method

2.1. Sample preparation and mounting

Seedlings of Arabidopsis thaliana expressing a cytosolic calcium indicator, the Yellow Cameleon NES-YC3.6, were grown on the top of fluorinated ethylene propylene (FEP) tubes filled with a jellified medium (GelriteTM at 0.5 % (v/v)). Cameleons are Förster Resonant Energy Transfer (FRET)-based calcium indicators, in which two fluorescent proteins, CFP and cpVenus are linked together by the calcium-binding protein Calmodulin. When the calcium concentration rises, the protein modifies its conformation and the FRET efficiency changes. The seedlings grow freely inside the gel in the pipette tips, kept in boxes filled with a hydroponic solution for 7 days. During imaging, tubes are transferred in an imaging chamber filled with the same medium and held vertically, without handling the hairs.

2.2 Light-sheet fluorescence microscope

* Corresponding author: andrea1.bassi@polimi.it

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).
The setup is a single-side illumination LSFM [2]. The light-sheet excitation is created by focusing the light of a single-mode fiber-coupled laser emitting at 445 nm with a cylindrical lens. A 10X water-dipping objective (UMPLFLN 10XW, Olympus) projects the generated 3.6 µm-thick light sheet on the sample. In this way, only the volume that is then observed with an orthogonally held water-dipping objective (UMPLFLN 10XW or UMPLFLN 20XW, Olympus) is excited, providing sectioning capability and limited phototoxicity. For the detection, custom-built image splitter creates 2 replicas of the same image with a different spectral content on the sensor of a fast camera (Neo 5.5 sCMOS, ANDOR). To this purpose, a dichroic filter at 505 nm (DMLP505, Thorlabs) and two broadband mirrors (BBSQ1-E02, Thorlabs) send the two replicas of the image through two band-pass filters (MF479-40 and MF535-22, Thorlabs) and a tube lens (UTLU-1-2, Olympus). This enables the immediate computation and visualisation of the FRET ratio (calculated as the cFP Venus signal divided by the CFP one) in real time, and hence of the calcium concentration variation. The acquisition is synchronised with the movement of a translation stage, which by moving the sample through the light sheet allows for the acquisition of multiple images of the root at variable depths. Volumes of 50 µm are scanned by sampling the root every 3 µm. Timelapses are acquired by collecting one timepoint every 3 s. An average of 5 root hairs per experiment are imaged over a population of at least 10 plants per type.

2.3 Analysis pipeline

To reduce the amount of data, we save only the maximum intensity projection of the ratio stack for each timepoint. For the fluorescence intensity extraction, we created our easy-to-use plugin for napari, a multi-dimensional image viewer for Python which allows to visualize and analyse large multi-dimensional images (available on the napari platform under the name napari-roi-registration). The plugin allows simultaneous registration of multiple regions of interest (ROIs) and subsequent extraction of information related to the ROIs. The plugin is divided in three widgets. The Background Widget allows for the removal of the background from each image of a stack. The Registration Widget provides simultaneous registration of a user-defined non-limited number of ROIs on the entire stack. Finally, the Processing Widgets allows one to extract the intensity and displacement of the registered ROIs, which is then shown in a plot. In addition, for oscillating behaviours like the one of root hairs, the Fourier transform of the signals can also be computed.

3 Results and discussion

The method allowed for the observation of calcium signals highly localised at tip of the root hairs that oscillate along time. To note that these oscillations have a characteristic high frequency, that can be extracted with a Fourier transform approach and have been estimated to be in average 0.036 Hz (1 every 28 s), overlapped to a lower frequency corresponding to an oscillation period of 104 s (Fig. 1). Mutant plants, in which genes under study were knocked-out, showed a different root hair phenotype and a disrupted oscillatory behaviour in the calcium gradient when such genes were involved in the healthy growth of the root hairs (Fig. 2). Through the in-depth analysis of calcium signals and other growth parameters, such as speed of growth and maximum length reached by the hairs, a better understanding of the role of the specific genes can be obtained.

References

2. A. Costa, A. Candeo, L. Fieramonti, G. Valentini, A. Bassi, PLOS ONE 10, 8 (2013)