Journal of Trace Elements in Medicine and Biology 47 (2018) 45–52

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Biochemistry

Rough and tough. How does silicic acid protect horsetail from fungal infection?

Gea Guerriero[a], Chinnoi Law[b], Ian Stokes[b], Katie L. Moore[c], Christopher Exley[b,*]

[a] Environmental Research and Innovation Department, Luxembourg Institute of Science and Technology, Esch/Alzette, Luxembourg
[b] The Birchall Centre, Lennard-Jones Laboratories, Keele University, Stoke-on-Trent, UK
[c] School of Materials and Photon Science Institute, University of Manchester, Oxford Road, Manchester, UK

ARTICLE INFO

Keywords:
Biogenic silica
NanoSIMS
Silicic acid
Callose
Horsetail
Fungal infection

ABSTRACT

Horsetail (*Equisetum arvense*) plants grew healthily for 10 weeks under both Si-deficient and Si-replete conditions. After 10 weeks, plants grown under Si-deficient conditions succumbed to fungal infection. We have used NanoSIMS and fluorescence microscopy to investigate silica deposition in the tissues of these plants. Horsetail grown under Si-deficient conditions did not deposit identifiable amounts of silica in their tissues. Plants grown under Si-replete conditions accumulated silica throughout their tissues and especially in the epidermis of the outer side of the leaf and the furrow region of the stem where it was continuous and often, as a double layer suggestive of a barrier function. We have previously shown, both *in vivo* (in horsetail and thale cress) and *in vitro* (using an undersaturated solution of $Si(OH)_4$), that callose is a "catalyst" of plant silica deposition. Here we support this finding by comparing the deposition of silica to that of callose and by showing that they are co-localized. We propose the existence of a synergistic mechanical protection by callose and silica against pathogens in horsetail, whereby the induction of callose synthesis and deposition is the first, biochemical line of defence and callose-induced precipitation of silica is the second, adventitious mechanical barrier.

1. Introduction

Silicon is a non-essential element for plants, as its presence is not required for the completion of their life cycle. Nevertheless, silicon improves plant vigour and resistance to biotic and abiotic stressors [1]. Plants take up silicon in the form of silicic acid, $Si(OH)_4$, deposit it as biogenic silica and are classified according to their propensities to accumulate it in their tissues. Horsetail and the commelinoid monocot rice are emblematic examples of highly silicifying plants (accumulating up to 10% silica by dry weight), while tomato is an excluder [2]. The association of biogenic silica with plant cell walls provides mechanical defence against pathogens [3] and is a deterrent against phytophagous insects [4].

A role for cell wall mixed-linkage glucans in biosilicification was shown in both horsetail [5] and rice [6]. In rice, the overexpression of a (1;3,1;4)-β-D-glucanase impacted the mechanical properties of the leaf blade and altered the distribution profile of silica [6]. In horsetail, it was demonstrated *in vivo* that silica accumulation mirrored callose deposition [7]. Importantly, this result was validated *in vitro*, where callose catalysed the precipitation of amorphous silica from an undersaturated solution of silicic acid [7]. Further support for a role of callose in templating silica deposition came in the non-Si accumulator thale cress, where mutants lacking the callose synthase gene *PMR4* showed significantly less silica deposition than either wild type or plants overexpressing this gene [8].

Using mild extraction procedures where silica was released from cell walls, silica was proposed to be involved in enhanced mechanical rigidity/stability against (a)biotic stresses in *Equisetum arvense* [9].

In this study, we provide evidence for the existence of a continuous silica layer in *E. arvense* tissues (double in specific regions), using for the first time NanoSIMS and propose a synergistic role with callose protecting against biotic stress.

2. Materials and methods

2.1. Hydroponic culture

Horsetail plants were collected, locally, from the wild in the early spring. Each plant had ca 3 cm of intact basal stem associated with the roots. The roots of washed plants were submerged in 20 mL of 1/6 Murashige Skoog (MS) basal salt growth solution (Sigma Aldrich M5524) at pH 5.8. The growth solutions were controlled to contain

* Corresponding author at: The Birchall Centre, Lennard – Jones Laboratories, Keele University, Staffordshire, ST5 5BG, UK.
E-mail address: c.exley@keele.ac.uk (C. Exley).

https://doi.org/10.1016/j.jtemb.2018.01.015
Received 7 December 2017; Received in revised form 25 January 2018; Accepted 26 January 2018

either 2 mM silicic acid (Alfa Aesar, Na_4SiO_4, Mw184), referred to as Si-replete, or 8 mM sodium (AnalaR BDH Labs, NaOH, Mw 40) referred to as Si-deficient. The growth environment consisted of ca 14 h light/10 h dark, at 25 °C. The growth solutions were replenished every other day. Plants were grown under these conditions for 12 weeks. The total Si content of the Si-deficient treatment was 12 µM as measured by TH GFAAS [7].

2.2. Preparation of plant tissues for PDMPO fluorescence

Horsetail samples were separated according to their anatomical region of basal stem, distal stem, leaves, nodes and root. Samples were cut with small scissors to a length of 1 cm. The samples (< 0.5 g) were digested in PFA Teflon© vessels with venting plugs and screw caps (CEM Microwave Technology Ltd, UK) using a 1:1 mixture of 15.8 M HNO_3 and 18.4 M H_2SO_4. Vessels were placed in insulating sleeves on a turntable, capable of holding up to 40 vessels. The microwave digestion programme was set up with Mars Xpress Microwave (CEM Microwave Technology Ltd, UK) using a CEM-provided Tissue Express organics method. Digested samples were diluted with ultrapure water (cond. < 0.067 µS/cm) and silica was collected by filtration (Whatman 0.45 µm filter paper) using several volumes of ultrapure water to rinse and clean the silica samples. Filter papers were placed in Petri-dishes in an incubator to dry. Collected silica was then weighed.

2.3. PDMPO fluorescence microscopy

Silica was immersed in 20 mM PIPES buffer at pH7 adjusted with dilute NaOH (Acros Organics, Mw 302.35) with 0.125 µM PDMPO (LysoSensor Yellow/blue DND-160 1 mM in DMSO). After 24 h incubation, 50 µL of the silica/PDMPO preparation was added to a cavity slide, covered with a cover slip and viewed using an Olympus BX50 fitted with a BXFLA fluorescent attachment using a U-MWU filter cube (Ex: 333–385 nm; Em: 400–700 nm). A ColourView III digital camera (OSIS FireWire Camera 3.0 digitizer) was used to capture images in conjunction with CELL* Imaging software (Olympus Cell* family, Olympus Soft Imaging solutions GmbH 3.0).

2.4. Preparation of plant tissues for NanoSIMS

Small sections of horsetail (< 1 mm thickness) were cut by hand with a scalpel and fixed in 0.1 M Na-cacodylate buffer (pH 7.4) with 3% glutaraldehyde. After fixation, samples were dehydrated in a graded ethanol series and infiltrated with increasing concentrations of LR White resin in ethanol. After polymerization, thin sections (1 µm) were cut on a microtome with a diamond knife, placed onto a droplet of water on platinum-coated Thermanox coverslips and stretched flat on a hotplate. Sections were coated with 10 nm of platinum to avoid charging in the NanoSIMS.

2.5. NanoSIMS

High resolution SIMS analysis was carried out on a Cameca NanoSIMS 50 using a 16 keV Cs^+ ion beam focused to approximately 100 nm with a beam current of 1.2-1.4 pA. Negative secondary ions generated during this process were analysed according to their mass to charge ratio using a double focusing mass spectrometer. The five detectors were precisely tuned using standards of Si and GaP to detect $^{12}C^-$, $^{12}C^{14}N^-$, $^{28}Si^-$, $^{31}P^{12}C^-$ and $^{32}S^-$ taking care to avoid mass interferences. The ion-induced secondary electron signal was also detected. For each area, a dose of 1×10^{17} Cs^+ ions cm^2 was implanted by continuously scanning a large defocused beam to remove the platinum coating and maximize signal intensity. Dwell times were 10 ms per pixel and for each region of interest.

2.6. Callose immunofluorescence

Identification of callose by immunofluorescence and fluorescence microscopy was carried out according to Pendle and Benitez-Alfonso (2015) [10] and briefly described herein. Small sections of horsetail tissues (< 1 mm thickness) were cut by hand with a scalpel, fixed and the cellulose in cell walls digested using 1% cellulase (Onozuka R-10, Yakult Pharm. Japan). Callose detection was performed on the extracted digested tissue using a (1–3)-β-glucan antibody (1:40; Biosupplies) and a secondary anti-mouse IgG-FITC antibody (1:40). Finally we used a Hoechst 33258 DNA counterstain and samples were mounted on glass slides and cover slipped. Tissue sections were viewed with a Zeiss Axioplan microscope (Blue Filter Cube #487910; Ex: 450–490 nm; Em: 515–565 nm) and images were captured using a Zeiss Axiocam MRc5 digital camera.

3. Results and discussion

3.1. Si-deficient horsetail succumbed to biotic stressors after 10 weeks of healthy growth

Horsetail (*Equisetum* sp.) is known to accumulate silica in its tissues [11]. However we have shown that it is not required for the growth of healthy plants [7].

While growing horsetail (*E. arvense*) for an investigation into the reversibility of biological silicification, we made a novel observation in relation to the resistance of horsetail to biotic stressors. Hydroponic culture in 1/6th MS under Si-replete (2 mM) or Si-deficient (12 µM) conditions (see Section 2) resulted in healthy looking horsetail plants, with the only difference being that plants grown in the presence of added silicon (4 plants) were rough to the touch, which we assumed reflected the deposition of silica in their tissues. After 10 weeks of apparently healthy growth, a change was observed in the turgor, which was reduced, and the colour, which became paler, of horsetail plants growing under Si-deficient conditions (4 plants) and these changes were coincident with visible signs of fungal infection in all 4 plants (Fig. 1). These observations are in agreement with what was previously shown in the literature, i.e. that Si-deficient horsetail had fragile, weak stems which subsequently withered, while Si-supplemented *Equisetum* did not [12]. Within 2 weeks, the infected areas were completely necrotic. Notably, this infection did not spread to horsetail plants grown in Si-replete conditions, despite the plants from different treatments being immediately adjacent to each other. Herein was evidence of the apparent benefit of silicon in protecting against fungal infection in horsetail. We endeavoured thereafter to establish how this apparently complete protection against the development of fungal infection was afforded, by investigating silica deposition in tissues of resistant plants using complementary imaging techniques.

3.2. NanoSIMS analysis of silica in horsetail tissues

The use of high-resolution secondary ion mass spectrometry (NanoSIMS) in plant biology couples high spatial resolution with sensitivity. Despite the complicated sample preparation protocol, this technique has been used to understand the distribution of several elements, including Si, in plant tissues [13,14].We used NanoSIMS (which identifies silica as $^{28}Si^-$ and hereafter will be referred to as silica) and fluorescence microscopy to map the exact location of silica in horsetail tissues. In particular we wanted to check for the presence of a silica-layer in horsetail tissue, since it is reported that one of the protective effects of silicon against pathogens is the formation of an “armour” providing mechanical shielding of cells [4]. This barrier is formed by the association of silica with cell wall components [15–17]. A previous study in the literature investigated the distribution of silica in horsetail using Raman imaging and highlighted its occurrence in the knobs and in a thin layer below the cuticle [18]. Our NanoSIMS analysis confirms