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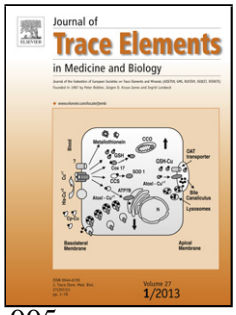

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Callose-associated silica deposition in Arabidopsis

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Abstract

The mechanism of biological silicification in plants remains to be elucidated. There are strong arguments supporting a role for the plant extracellular matrix and the β-1-3-glucan, callose, has been identified as a possible template for silica deposition in the common horsetail, *Equisetum arvense*. The model plant *Arabidopsis thaliana,* which is not known as a silica accumulator, can be engineered to produce mutants in which, following a pathogen-associated molecular pattern challenge, callose production in leaves is either induced (*35S::PMR4-GFP*) or not (*pmr4*). We have grown these mutants hydroponically in the presence of added silicon to test if the induction of callose results in greater silica deposition in the leaves. Callose induction was identified throughout leaf tissue of wild type Arabidopsis and the mutant *35S::PMR4-GFP* but not in the mutant *pmr4*. Similarly both wild type Arabidopsis and the mutant *35S::PMR4-GFP* showed extensive silicification of leaf tissue while the *pmr4* mutant deposited very little silica in its leaf tissues. Wild type Arabidopsis and the mutant *35S::PMR4-GFP* responded to a pathogen-like challenge by producing both callose and biogenic silica coincidently in their leaf tissues. Trichomes in particular showed both callose deposition and extensive silicification. The lack of both induced callose deposition and subsequent silicification in the *pmr4* mutant strongly suggested that the biochemistry of callose formation and deposition were allied to biological silicification in Arabidopsis.

Keywords: Biological silicification, biogenic silica, callose, Arabidopsis, aniline blue, PDMPO fluorescence.

Introduction

All plants take up silicic acid ($Si(OH)_4$) via their roots and transport it throughout the tissues following water [1]. However, not all plants deposit $Si(OH)_4$ as biogenic silica to the same degree with some plants such as Equisetum (horsetails) being considered as silica accumulators with as much as 5% of their tissue dry weight being attributed to biological silicification [2]. The biochemical machinery which differentiates silica accumulators such as rice and horsetail from non-accumulators such as Arabidopsis remains to be understood and is the subject of a significant research effort. Of particular interest is the plant extracellular matrix as a factor in templating biogenic silica deposition [2] and we have identified the β-1-3-glucan callose as a biomolecule involved in silica deposition in horsetail [3]. We were able to show that not only does silica deposition in horsetail mirror callose deposition but also that *in vitro* callose could induce the formation of silica from an under-saturated solution of $Si(OH)_4$. To test a role for callose in biological silicification we obtained seeds of wild type Arabidopsis and two mutants with differing capabilities with respect to stress-induced callose formation [4]. The hypothesis being that under identical conditions of availability of $Si(OH)_4$ there would be significantly less silica deposition in the mutant engineered to resist callose induction (*pmr4*) than one engineered to show increased callose synthase activity (*35S::PMR4-GFP*).

Materials and methods

Hydroponic culture of Arabidopsis Seeds of Arabidopsis (*Arabidopsis thaliana*) wild type (Columbia) and *pmr4* (allele 1) and *35S::PMR4-GFP* transgenic plants were kindly provided by CA Voigt [4]. Seeds were germinated in the dark on 1% agar contained within the lid of a punctured Eppendorf tube. The lids are placed in a tube rack which in turn is placed in a tank filled with ¼ strength MS medium. Two growth mediums were used, one with (+Si) and one

without (-Si) added Si at 2 mM, at pH 5.80±0.05. The latter medium (-Si) included an additional 8 mM Na^+ to account for Si addition as Na_4SiO_4. After 2 weeks, during which time roots have traversed agar plugs and entered the growth medium, the Eppendorf lids were placed on a polystyrene support floated on the appropriate growth medium (Figure 1a). After a further 3 weeks of a 14h light / 10h dark cycle at 25°C growth media were supplemented with 35 mg/L chitosan, an elicitor mimicking fungal infection, and known to induce the formation of callose [5], and grown for an additional 2 weeks. At this point all plants are harvested for examination of both presence of tissue callose and silica deposition.

Identification of callose in tissues We used an established method for the identification of callose in plant tissue using aniline blue [6]. Briefly, leaves from plants from each group are detached and fixed and destained in a 1/3 acetic acid/ethanol solution until approximately transparent. Leaves are then washed for 30min. in 150 mM Na_2HPO_4 and then incubated for 2h in 150mM Na_2HPO_4 which included 0.01% aniline blue. Images of callose were obtained 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). Callose was identified as distinct green fluorescence.

Digestion of plant tissue Leaves from plants from each group were detached and dried to a constant weight in an incubator at 37°C at which point 0.1 g of each were placed in acid-washed 20 mL PFA teflon© vessels. The samples were then digested in a 1:1 mixture of 15.8M HNO_3 and 18.4M H_2SO_4 using a Mars Xpress microwave oven (CEM Microwave Technology Ltd.). The acid digests were clear and, upon dilution with 8 mL of ultrapure water, were filtered and the residues washed several times with further volumes of ultrapure

water. Filters were then placed in plastic Petri dishes in an incubator at 37°C to achieve dryness over several days. Dry residues off each filter were then collected into Bijoux tubes and stored in a dry, sealed, Perspex cabinet.

PDMPO labelling of Arabidopsis-derived silica We used an established method for the identification of biogenic silica in plant tissues [3]. Briefly, silica residues collected from filters were suspended in 20 mM PIPES at pH 7 and containing 0.125μM 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)-methoxy) phenyl) oxazole (PDMPO; LysoSensor Yellow/Blue DND-160, 1 mM in DMSO). Suspensions were left for 24h to allow the reaction between silica surfaces and PDMPO to achieve completion after which 50 μL samples were transferred to a cavity slide 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).

Results and discussion

Germination and plant growth There were no differences in germination and plant growth between those treatments which were (+Si) or were not (-Si) supplemented with silicon. However, wild type seeds germinated better than seeds of either of the transgenic plants. Similarly, wild type plants grew larger than either *pmr4* or *35S::PMR4-GFP* plants though all plants appeared healthy even following challenges with chitosan (Figure 1b).

Identification of callose in leaves Wild type plants grown in both Si treatments and subsequently challenged with chitosan showed significant positive callose fluorescence on

the leaves and especially associated with trichomes and mesophyll tissue (Figure 2a,b). Some callose fluorescence was associated with venation. A high level of positive callose fluorescence associated with similar areas of the leaf was also seen in the *35S::PMR4-GFP* plants (Figure 2c,d). The *pmr4* plants showed no positive callose fluorescence except a very small amount associated with venation (Figure 2e,f).

Identification of silica deposition in leaves Biogenic silica was only observed in plants grown in treatments supplemented with silicon (+Si). Wild type plants challenged with chitosan showed significant, up to 0.5% silica/dry weight of leaf tissue, and extensive silica deposition in leaves. Trichomes were quite heavily silicified as were significant areas of mesophyll tissue (Figure 3a,b). A similar degree of silica deposition was also observed in the *35S::PMR4-GFP* plants with many silicified trichomes being collected from leaf digests (Figure 3c,d). In comparison hardly any silica was collected from digests of leaves of the *pmr4* plants with only silica remnants being identified by PDMPO fluorescence (Figure 3e,f).

We have tested the hypothesis that deposition of biogenic silica will be significantly enhanced in a non-silica accumulator which has been engineered to induce callose formation following a challenge with a pathogen-associated molecular pattern (PAMP). We grew hydroponically (i) wild type *Arabidopsis thaliana*, (ii) the *pmr4* mutant which is expected not to produce PAMP-induced callose and (iii) the *35S::PMR4-GFP* mutant which is expected to produce significant PAMP-induced callose and challenged all plants with chitosan. Induction of callose deposition in leaves was identified qualitatively using aniline blue fluorescence. Wild type plants showed callose deposition throughout tissues and especially in the mesophyll and associated with trichomes (Figure 2a,b). Callose deposition was similar in the leaves of the *35S::PMR4-GFP* mutant (Figure 2c,d) though perhaps more extensive than in wild type. Callose deposition was almost completely absent in the *pmr4* mutants except for small amounts associated with venation (Figure 2e,f). There were no substantive differences

in callose deposition between plants grown in silicon-supplemented (+Si) and non-silicon-supplemented (-Si) growth media. The identification of silica deposition in the leaves of wild type and mutant Arabidopsis was achieved qualitatively using PDMPO fluorescence. Microwave-assisted acid digestion of plant tissue only leaves silica as a residue and when this is collected by filtration it can be viewed using fluorescence microscopy. A significant amount of biogenic silica, up to 0.5% of dry weight of tissue, was collected from the leaves of wild type Arabidopsis. Silicified structures were identified as trichomes, parenchyma and, occasionally, epidermal cells as well as other unidentifiable remnants (Figure 3a,b). Biogenic silica was similarly abundant in digests of the *35S::PMR4-GFP* mutants where silicified trichomes were especially represented (Figure 3c,d). While both types of plant which demonstrated significant deposits of callose additionally showed relatively high levels of biological silicification the *pmr4* plants showed neither significant deposits of callose nor extensive biological silicification. Arabidopsis has 12 genes encoding callose synthase with PMR4 (the subject of this study) being associated with induction of callose synthase following abiotic or biotic stress [4]. Therefore callose was identified associated with venation in the *pmr4* mutants and likewise remnants of silica were obtained from digests of leaves of *pmr4* mutants some of which had the appearance of parts of trichomes but these silica deposits were rare and difficult to find using fluorescence microscopy (Figure 3e,f). We have been able to show a clear if qualitative relationship between the induction of callose and deposition of biogenic silica in Arabidopsis, a plant not known for accumulating significant amounts of silica in its leaves. Our results for callose induction strongly support research identifying a role for callose in resistance to powdery mildew in Arabidopsis [4] while our findings for silica deposition suggest a mechanism for silicon-induced resistance to the same pathogen in Arabidopsis [7,8]. We reported previously that callose-associated silica deposition provided protection against powdery mildew in *Equisetum arvense* (horsetail) and

we speculated that biogenic silica presented a physical barrier to the entry of the pathogen [3]. The coincident deposition of biogenic silica and callose in Arabidopsis may provide resistance to powdery mildew by a similar mechanism. In horsetail we were able to demonstrate almost mirror-like depositions of callose and silica during the development of stomata [3]. Herein we have seen similarities with the development of trichomes with silica deposition appearing to mirror the role of callose in the development of these epidermal hair cells [9] (Figure 4). Silicification in the *35S::PMR4-GFP* mutants was particularly evident in the apical part of trichomes above the Ortmannian ring (Figure 3c,d) which is known to be rich in callose [9]. Biogenic silica is clearly intimately associated with the machinery of the plant extracellular matrix [2] and almost certainly adventitiously so. However, future experiments will be required to confirm the nature of these relationships and whether callose and similar biomolecules are templates for biological silicification more generally.

Conclusions

We have shown that induction of callose synthesis resulted in increased deposition of biogenic silica in Arabidopsis. The results support our previous observation of a link between callose and biological silicification in Equisetum and they add further weight to the suggestion that callose acts as a template for the deposition of biogenic silica in plants and algae.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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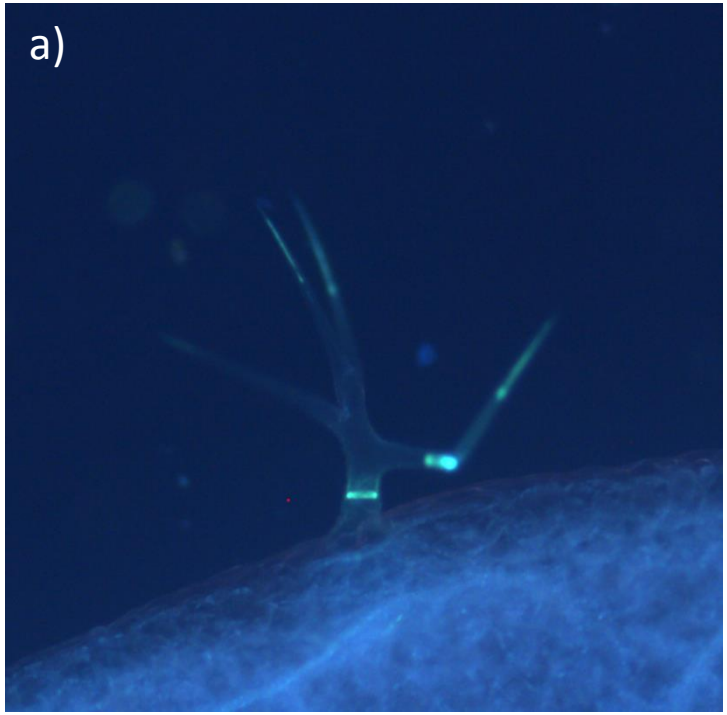
Figure Legends

Figure 1 a. Schematic of the experimental apparatus for hydroponic culture of Arabidopsis. **b.** Experimental set-up showing 5 week old Arabidopsis prior to harvesting.

Figure 2 Fluorescence imaging of aniline blue staining of callose in leaves of Arabidopsis. Callose is identified as bright green fluorescence. a,b. Wild type, callose fluorescence is found associated with trichomes (a) and mesophyll tissue (b); **c,d.** Extensive deposition of callose throughout leaf tissues in the mutant *35S::PMR4-GFP*; **e,f.** Callose fluorescence only seen associated with venation in the *pmr4* mutant. Scale bars are all 500μm.

Figure 3 PDMPO fluorescence of silica isolated from digests of leaves of Arabidopsis. a,b. Wild type showing extensive silicification of leaf tissue (a) and specifically associated with trichomes and mesophyll (b); **c,d.** Many heavily silicified structures in leaves taken from the *35S::PMR4-GFP* mutant (c) and especially trichomes (d); **e,f.** Very limited evidence of silica deposition in the *pmr4* mutant just small remnants of structures including trichomes. Scale bars are 500μm for a,c,e,f and 200μm for b,d.

Figure 4 The colocalisation of callose (a) and silica (b) in trichomes.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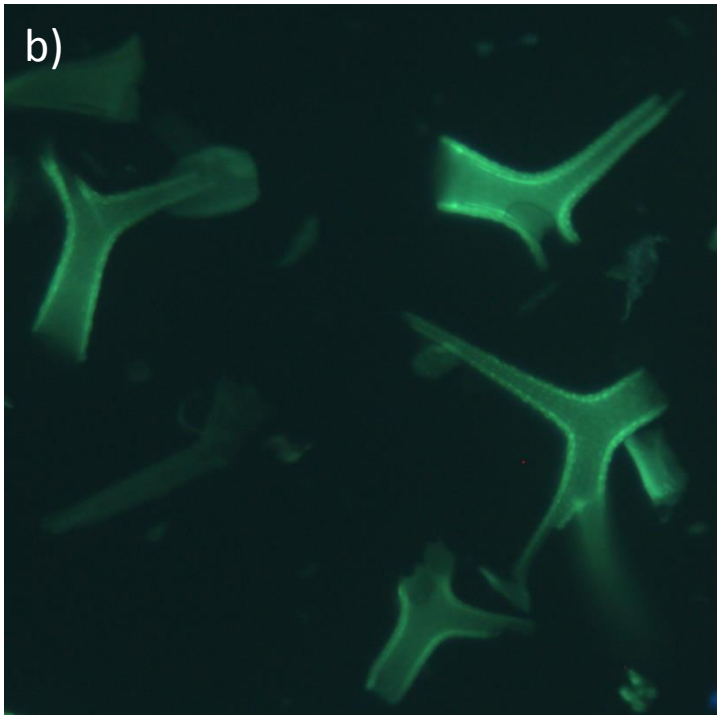
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