pH measurement of tubular vacuoles of an arbuscular mycorrhizal fungus, *Gigaspora margarita*

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Abstract Arbuscular mycorrhizal fungi play an important role in phosphate supply to the host plants. The fungal hyphae contain tubular vacuoles where phosphate compounds such as polyphosphate are accumulated. Despite their importance for the phosphate storage, little is known about the physiological properties of the tubular vacuoles in arbuscular mycorrhizal fungi. As an indicator of the physiological

state in vacuoles, we measured pH of tubular vacuoles in living hyphae of arbuscular mycorrhizal fungus *Gigaspora margarita* using ratio image analysis with pH-dependent fluorescent probe, 6-carboxyfluorescein. Fluorescent images of the fine tubular vacuoles were obtained using a laser-scanning confocal microscope, which enabled calculation of vacuolar pH with high spatial resolution. The tubular vacuoles showed mean pH of 5.6 and a pH range of 5.1–6.3. These results suggest that the tubular vacuoles of arbuscular mycorrhizal fungi have a mildly acidic pH just like vacuoles of other fungal species including yeast and ectomycorrhizal fungi.

Keywords 6-carboxyfluorescein diacetate · arbuscular mycorrhizal symbiosis · laser scanning confocal microscopy · tubular vacuole · vacuolar pH

Introduction

Arbuscular mycorrhizal (AM) fungi belonging to the phylum Glomeromycota are obligate symbionts of most land plants. The AM fungi colonizing plant roots depend on their hosts for carbon. In return, the AM fungi uptake inorganic phosphate (P_i) from the soil through the extraradical hyphae and provide them to the host plants via arbuscules formed in the plant cortical cells (Smith and Gianinazzi-Pearson 1988; Harrison 1999; Smith and Read 2008).

The P_i taken by the extraradical hyphae had been regarded as accumulated in spherical vacuoles as a form of polyphosphate granule and translocated in the aseptate hyphae by cytoplasmic streaming (Cox et al. 1980). However, the vacuolar system in living filamentous fungi has been revealed as a complex tubular system, not a simple spherical form (Shepherd et al. 1993; Rees et al. 1994; Uetake et al. 2002; Shoji et al. 2006; Ashford and Allaway 2007). The vacuoles of ectomycorrhizal (EM) fungi are motile and consist of tubular and spherical forms (Shepherd et al. 1993; Cole et al. 1998). The motile tubular vacuole system is probably involved in long distance P_i translocation (Ashford et al. 1994; Darrah et al. 2006). In living AM fungi, extensive tubular vacuole systems occur in bundles and occupy most of the cell volume (Uetake et al. 2002). The tubular vacuoles contain polyphosphate in dispersed form, which is a linear polymer of orthophosphate, indicating that the vacuole is an important organelle to store phosphate compounds and possibly to translocate polyphosphate in AM fungal hyphae (Kuga et al. 2008). Despite their importance, physiological properties of the AM fungal tubular vacuoles are less understood.

Intracellular pH is an important physiological parameter. Cytosolic and organelle pH influences on protein stability, enzyme and ion channel activity, and many other physiological processes. Using ratio metric imaging of pH indicator BCECF, Jolicoeur et al. (1998) estimated that the cytoplasmic pH of AM fungus *Gigaspora margarita* and *Glomus intraradices* was nearly neutral, although the cytosolic pH profile varied somewhat along hyphae. In contrast to the cytosolic pH, almost

nothing is known about pH of tubular vacuoles in AM fungi. In yeast, spherical vacuoles show mildly acidic pH of approximately 6.2–6.5 (Makarow and Nevalainen 1987; Preston et al. 1989). Similarly, pH of tubular vacuole systems of EM fungus *Pisolithus tinctorius* have mild acidity, whereas the vacuolar pH exhibited widely varied pH of 4.3–7.5 based on ratio imaging of 6-carboxyfluorescein (CF)-loaded vacuoles (Rost et al. 1995). Using ^{31}P-NMR spectroscopy, acidic compartments of extraradical hyphae in AM fungus *G. intraradices* were estimated as pH 6.0 based on the chemical shift of terminal phosphate residues in the polyphosphate chain (Viereck et al. 2004). This result might indicate the acidity of tubular vacuoles in AM fungi, but limitations exist in spatial resolution of NMR spectroscopy in distinguishing individual tubular vacuoles of AM fungi.

Herein, we describe internal pH of individual tubular vacuoles in germ tubes of AM fungus *G. margarita* measured using ratio image analysis with pH-dependent fluorescent probe 6-CF. The CF does not easily permeate plasma membrane, although its derivative, non-fluorescent carboxyfluorescein diacetate (CFDA), is moderately permeant to the plasma membrane (Haugland 1996). When the CFDA is loaded to live cells, it is hydrolyzed to fluorescent CF by intracellular nonspecific esterases; it accumulates in vacuoles (Preston et al. 1989; Rost et al. 1995). For this study, fluorescent images of AM fungal vacuoles were acquired using a laser-scanning confocal microscope to acquire high-resolution images of fine tubular vacuoles in germ tubes. The germ tubes are useful for cytological studies because of easy handling of fresh materials under a microscope (Uetake et al. 2002; Saito et al. 2004). Their physiological properties and vacuolar organization are likely to resemble those of extraradical hyphae (Yao et al. 2010).

Materials and methods

Preparation of fungal materials

The isolate of *Gigaspora margarita* Becker & Hall (MAFF 520054) was used. The fungal spores were produced and extracted as described previously by Uetake et al. (2002). The spores were immersed in 2% chloramine T for 10 min. Then they were washed five times with sterilized distilled water. Autoclaved Long-Ashton (1.33 mM NaH_2PO_4, 5 mM NH_4NO_3, 2 mM K_2SO_4, 4 mM $CaCl_2$, and 1.5 mM $MgSO_4$; pH 5.8) with 1% agar medium was prepared in a plastic Petri dish under sterile conditions. Pieces of cellulose acetate membrane (5 × 10 mm, 0.2 µm pore size) were arranged on the surface of the medium. Four spores were placed on each membrane. The plates were inverted and incubated in the dark at 25°C for 10 days. Germ tubes on the membrane were used for pH measurement of vacuoles.

Loading of 6-CF

CFDA isomer free (Molecular Probes Inc., OR, USA) was dissolved in dimethylsulfoxide at a concentration of 1 mM and was stored at -20°C in the dark. For fluorescent labeling of living fungal vacuoles, the stock solution was diluted to 50 µM with 50 mM Tris-maleate buffer pH 5.5. Each membrane with germ tubes was transferred from the Petri dish to a drop of CFDA working solution on a glass slide. Under a dissecting microscope, drops of the fluorescent probe were added to the slide. Then the germ tubes were transferred carefully from the membrane to the slide using needles. The slide was incubated for 30 min at 32°C and then rinsed with distilled water. After sealing the edge of the cover slip with nail polish, the slide was observed using a laser-scanning confocal microscope.

Image acquisition

Fluorescence microscopy was performed using a laser-scanning confocal microscope (LSM510; Carl Zeiss Inc., Jena, Germany) with a plan Apochromat 63×/1.4 oil DIC objective (Carl Zeiss Inc.). Fluorescence of CF was excited by 458 nm and 488 nm using an argon laser. The emitted fluorescence was detected with 505–530 nm band pass filter. The detector gain level and amplifier offset level were optimized. The same parameters were used throughout the experiments. Images of CF-stained vacuoles were acquired as a 0.7 μm optical slice (94 μm pinhole size) at 1024 × 1024 pixels and 8 bits (256 grey levels) resolution. Fluorescent images at 458 nm and 488 nm excitation were acquired successively within 4 s. Measurements were conducted at approximately 25°C. In all, 91 tubular vacuoles from 9 germ tubes were examined.

Image analysis

Acquired images were exported in TIFF format using software (LSM 510 ver. 2.5; Carl Zeiss Inc.). A series of regions of interest (ROI) was defined in the central area of the vacuole on 458 nm excited images. The average fluorescence intensity, converted to 256 grey levels, was measured using Scion Image (Scion Corp., Maryland, USA). The corresponding region was measured on the 488 nm-excited images. The average background values were subtracted independently for each ROI at each image. Ratios of fluorescence with 488 nm excitation to that with 458 nm excitation were calculated. The following images of vacuoles were not further analyzed: those showing (i) saturated fluorescence intensity and (ii) very weak fluorescence intensity (grey level of less than about 70 in the 458 nm-excited images). The pH in individual vacuoles was calculated from the fluorescence ratio using the ratiometric pH calibration curve described below. The pH profile along the germ tube was expressed at 205 × 205 pixel resolution (5 × 5 binning image) with pseudo-colors, which show the pH values calculated from the ratiometric pH calibration curve.

Standardization

Solutions of CF (Molecular Probes Inc.) were prepared in 50 mM Tris-maleate buffer ranging from pH 4.2 to pH 8.9. Two-sided tape (1.5 × 1.5 mm 1.16 mm thickness; Cemedine Co. Ltd., Tokyo, Japan) with a hole in the center was put on a glass slide. Then 20 µl of CF solution was added to the hole. It was covered with a coverslip. The ratio of fluorescence for each pH was obtained as described above. A sigmoidal model was fitted to the data using software (DeltaGraph 5.4; Red Rock Software, Utah, USA): ratio = $a + b/[(1 + (pH/c)^d]$, where a, b, c, and d are the parameters to be estimated.

Results

An *in vitro* pH calibration curve was drawn for CF by calculating the fluorescence ratio of 488 nm excited and 458 nm excited images (Fig. 1). Images were acquired using a laser-scanning confocal microscope with fixed values of laser intensity, detector gain, amplifier offset, pinhole size and scanning speed parameters. These settings were also used for the image acquisition of AM fungal vacuoles. The ratiometric data were well fitted using a sigmoidal curve ($r^2 = 0.993$). The *pK*a for CF *in vitro* was 5.79, which is equivalent to the values (5.80 *in vitro*) reported by Preston et al. (1989). A study of yeast *Saccharomyces cerevisiae* found no substantial difference between *in vitro* and *in vivo* pH calibration curve of CF: *pK*a for CF of 5.80 *in vitro* and 5.84 *in vivo* (Preston et al. 1989). Therefore, the *in vitro* ratiometric pH calibration curve was used for vacuolar pH measurements in this study.

Loading with CFDA showed that *G. margarita* has tubular vacuoles in germ tubes (Fig. 2a). The vacuolar profiles resemble those stained with Oregon Green 488 carboxylic acid diacetate

(carboxy-DFFDA) (Uetake et al. 2002; Saito et al. 2004) that have been used as a selective probe for fungal vacuoles (Cole et al. 1998; Uetake et al. 2002). A pH-mapping image of tubular vacuoles in a *G. margarita* germ tube is presented in Fig. 2b, which was produced by calculating the ratios of fluorescent intensity of the 458 nm-excited and 488 nm-excited images. The tubular vacuoles in the germ tube were predominantly in the range pH 5.5–6.5. However, vacuolar fringes sometimes exhibited higher or lower pH than the central regions of the tubular vacuoles because the tubular vacuoles moved slightly during acquisition of the two images within 3–4 s. In the following experiments, overlap of vacuolar location between the two images was confirmed. Then the ROI for pH measurement was defined in the central area of the vacuoles (Fig. 3).

To evaluate the variation of vacuolar pH in a single germ tube, pH of tubular vacuoles in approximately 50 μm length of a germ tube was measured for nine germ tubes, each of which emerged from a different spore. The vacuolar pH variation was not so great in a single germ tube, but the mean pH values in a single tubular vacuole were 5.4–6.0 among the nine germ tubes (Fig. 4a). Combined data for vacuoles in the entire nine germ tubes showed that the vacuolar pH of *G. margarita* was mildly acidic, with pH of 5.1–6.3 (avg. 5.6; Fig. 4b).

Discussion

This report is the first to describe pH measurement of AM fungal tubular vacuoles using the pH-dependent fluorescent probe. Ratiometric pH measurements of fine tubular vacuoles with high spatial resolution were made possible using a laser-scanning confocal microscope. The vacuolar pH of *G. margarita* germ tubes measured in the present study was mildly acidic, with pH ranging from 5.1–6.3, which contrasts against neutral cytosolic pH 6.6–7.0 measured by pH-dependent probe BCECF

(Jolicoeur et al. 1998). The vacuolar pH (mean pH of 5.6) measured using CFDA was consistent with pH in a putative vacuolar compartment estimated from a chemical shift value of P_i signal by *in vivo* ^{31}P-NMR analysis of *G. intraradices*, showing pH 5.6 (Rasmussen et al. 2000) and 5.5 (Viereck et al. 2004). In yeast *S. cerevisiae*, vacuolar pH was found to be 6.2 (Preston et al. 1989) and 6.5 (Makarow and Nevalainen 1987) based on pH-dependence of fluorescent probes. Vacuolar pH of *Aspergillus niger* was maintained at constant values of 6.2 measured using ^{31}P-NMR when extracellular pH was varied between 1.5 and 7.0 (Hesse et al. 2002). Several strains of encapsulated yeast *Cryptococcus neoformans* exhibit vacuolar pH ranging from 5.3 to 5.9 with a mean of 5.6 (Harrison et al. 2002). It can be inferred therefore that fungal vacuoles might have mildly acidic pH. In the EM fungus *P. tinctorius*, two populations of vacuoles were recognized: one having a modal group pH 7.0–7.5 with pH of 4.3–7.5 in predominantly terminal cells, and the other vacuoles having a modal group pH 5.5–6.0 with pH of 4.8–7.2 in predominantly penultimate cells (Rost et al. 1995). Ashford (1998) suggested functional differentiation within the vacuole system similar to endosome–lysosome networks in mammalian cells. In the present work, pH measurements were done in regions that were distal from the hyphal tip because of the small number of hyphal tips where tubular vacuoles were very fragile. It was difficult to compare the vacuolar pH in between the hyphal tip and the other region. Therefore, variation of the vacuolar pH along the AM fungal hyphae cannot be excluded.

Vacuole acidification influences ion transport, protein sorting, proteolytic processing and regulation of cytoplasmic pH (Stevens and Forgac 1997; Ashford and Allaway 2007). The H^+ electrochemical gradient between vacuoles and cytosol is derived from the balance of H^+ loading by V-type H^+ ATPases and leakage across the vacuolar membrane. The V-ATPases are multiple complexes comprising V_0 and V_1 domains (Beyenbach and Wieczorek 2006). The V_0 domain consists of a multiple subunit complex to form an integral H^+ translocating channel. It is connected to a cytoplasmically located V_1 complex by which ATP is hydrolyzed to harness the energy for H^+ transport into a vacuole

lumen. It is particularly interesting that a *vma4* mutant strain of yeast, in which V-ATPase activity is completely deficient by deletion of subunit E of the V_1 domain (Zhang et al. 1998) and vacuolar pH is alkalized (Brett et al. 2011), was found to have no accumulation of polyphosphate (Ogawa et al. 2000). In *A. niger*, some evidence suggests that polyphosphate accumulation coincides with the increase of pH gradient between vacuole and cytoplasm (Hesse et al. 2002). Polyphosphate is known to be synthesized by the membrane-integral vacuolar transporter chaperone (VTC) complex from ATP in yeast (Hothorn et al. 2009). The V-ATPase activity or vacuolar pH might be involved indirectly in the polyphosphate accumulation in vacuoles via regulation of VTC activity. Cytochemical and biochemical reports have described that AM fungi also synthesize polyphosphate and accumulate it in tubular vacuoles (Cox et al. 1980; Kuga et al. 2008; Tani et al. 2009). The vacuoles in which polyphosphate accumulates have pH 6.0, as indicated by the chemical shift of terminal phosphate residues in the polyphosphate chain by *in vivo* ^{31}P-NMR spectroscopy (Viereck et al. 2004), which is consistent with our pH measurement of tubular vacuoles. In the mildly acidic condition of the tubular vacuoles, it was inferred that polyphosphate is partially charged and that it exists in soluble form because acid dissociation constants of polyphosphate (16 residues) are 5–6.5 (MacDonald and Mazurek 1987). Future work should specifically examine the relation between vacuolar pH and its physiological properties related to mineral storage and translocation in AM fungi.

Acknowledgements We thank Ryo Ohtomo, Tomoko Kojima, and Shotaro Ando (National Agriculture and Food Research Organization) for experimental support. We also thank Yukari Kuga (Hiroshima University) for critical reading of the manuscript. This work was supported in part by the Programme for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry to KS. This work was also supported in part by the Promotion of Basic Research Activities for Innovative Biosciences

(PROBRAIN) of the Bio-oriented Technology Research Advancement Institution of Japan, and for Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research (B) to MS.

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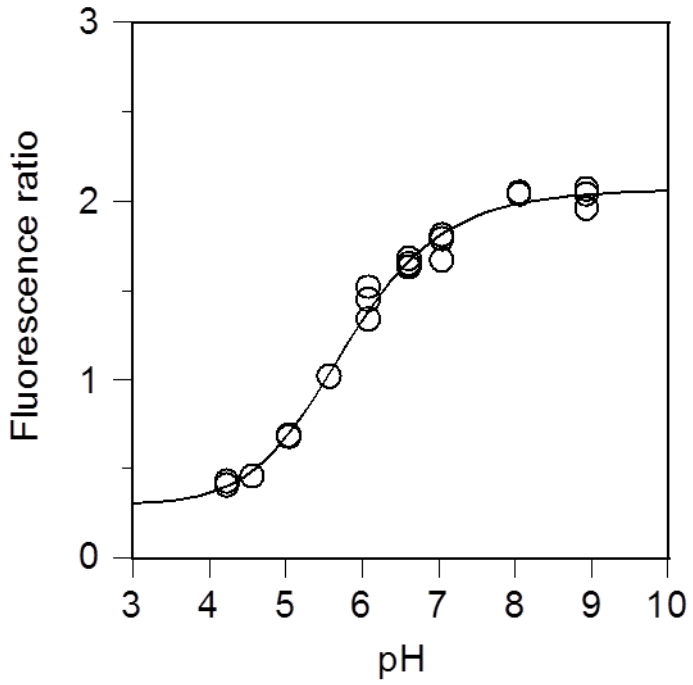

Fig. 1 *In vitro* standard pH calibration curve of 6-CF. The fluorescence ratio (open circles) of 488 nm- and 458 nm-excited images was calculated for each CF solution at various pH values. Solid line shows a sigmoidal curve fitted by the following equation: fluorescence ratio = 0.30 + 1.77/[(1 + (pH/5.79)$^{-9.05}$)] (r^2 = 0.993).

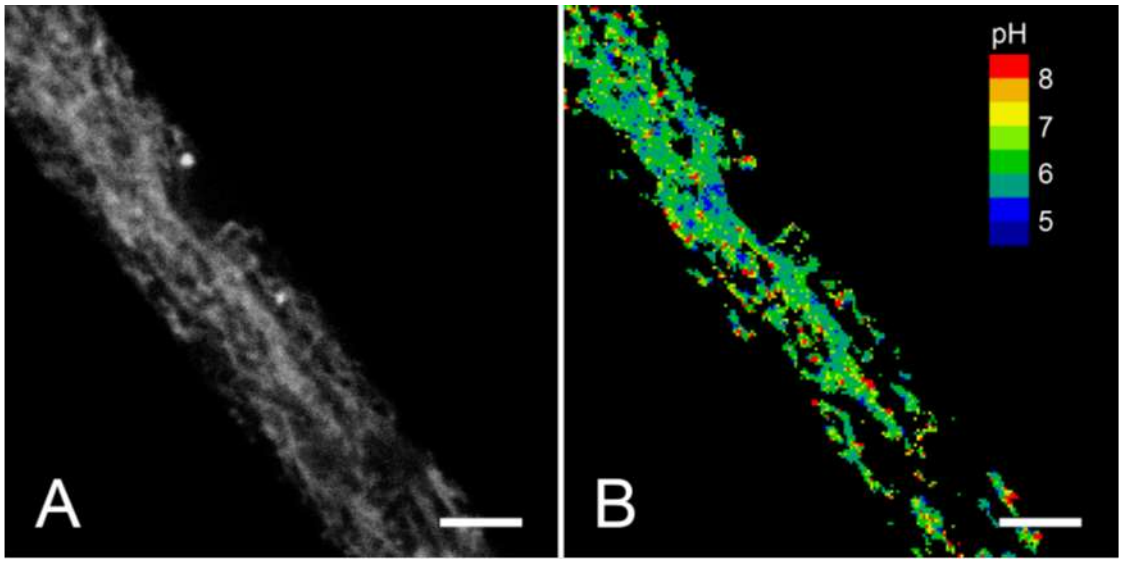

Fig. 2 Map of vacuolar pH within a living germ tube of *G. margarita*. **a** Fluorescent image of tubular vacuolar system stained with CFDA and visualized by 488 nm excitation. **b** Pseudo-colored image showing the two-dimensional pH distribution in the same germ tube calculated by the ratio image analysis as described in Materials and Methods. Bars: 5.0 µm.

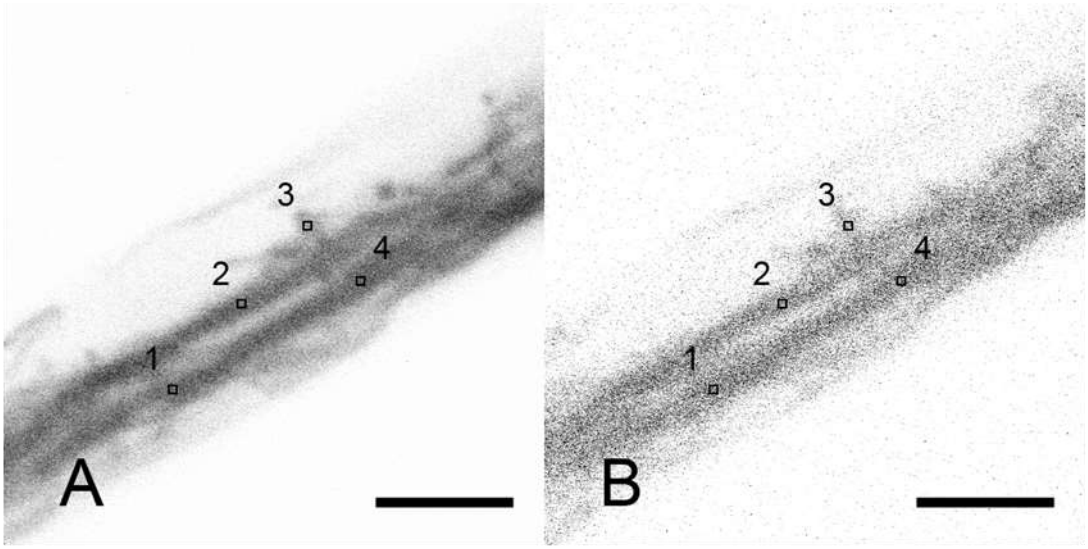

Fig. 3 Images of living germ tube of *G. margarita* stained with CFDA acquired by a laser-scanning confocal microscope. Fluorescent images of tubular vacuolar system are visualized by 488 nm excitation (**a**) and 458 nm excitation (**b**). Squares exhibit ROIs in which fluorescent intensity is measured. The ROIs located central region of tubular vacuoles. The same number exhibits the same location of a ROI between 488 nm- and 458 nm-excited images. ROI1: pH 5.9; ROI2: pH 6.1; ROI3: pH 5.9; ROI4: pH 5.8. Bars: 5.0 µm.

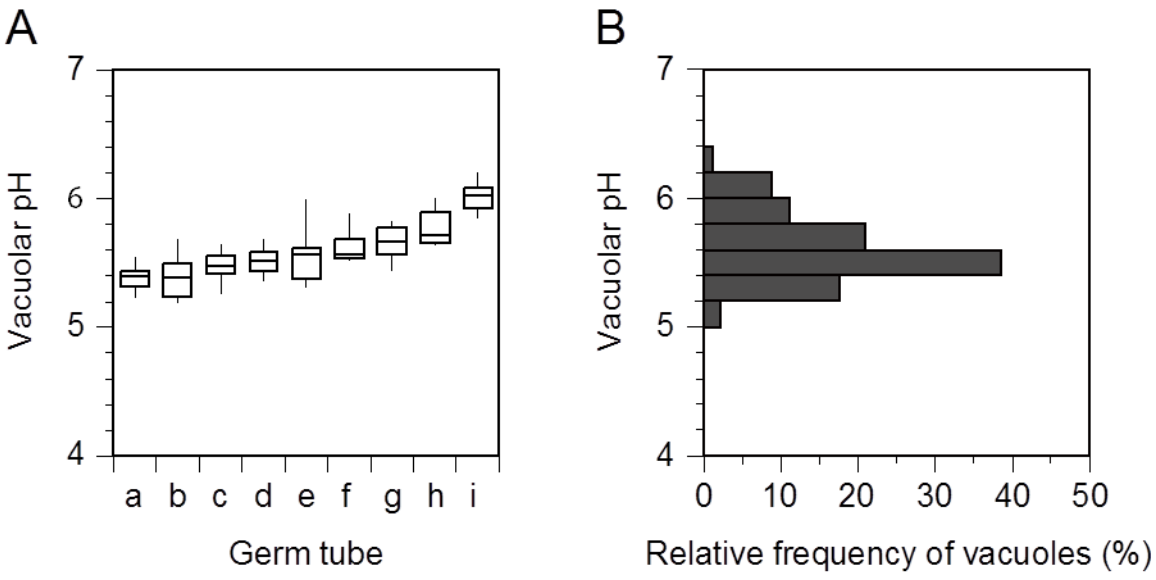

Fig. 4 Vacuolar pH distributions in germ tubes of *G. margarita*. **a** pH of 7-14 tubular vacuoles in a 36 × 36 µm^2 image were measured for each single germ tube. The box-plot represents the 25th, 50th and 75th percentile, the right whisker ranges from the 75th to the 90th percentile and the left whisker ranges from the 10th to the 25th percentile. **b** Frequency distribution of vacuolar pH in tubular vacuoles (n = 91) of 9 germ tubes.