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SHORT COMMUNICATION

Vacuolar occupancy is crucial for cell elongation and growth regardless of the underlying mechanism

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ABSTRACT

In the physiological range, the phytohormone auxin inhibits the growth of underground tissues. In the roots of *Arabidopsis thaliana*, cell size inhibition has been shown to be accompanied by auxin-mediated reduction of vacuole size. A tonoplast-localized protein family (Networked 4) with actin-binding capacity was demonstrated to modulate the compactness of the vacuole. Overexpression of NET4A led to smaller, more spherical and compact vacuoles, which occupied less cellular space compared to wild type. This reduction of vacuolar occupancy is similar to the observed auxin-induced decrease in occupancy, albeit there are enormous morphological differences. Here, we show that a *net4a net4b* double mutant and a NET4A overexpressor line are still sensitive to auxin-induced vacuolar constrictions. However, the overexpressor showed a partial auxin resistance accompanied by more compact vacuoles, thereby indicating an additional regulatory mechanism. Furthermore, we show that other NET superfamily members do not compensate for the loss of NET4A and NET4B expression on the transcriptional level. This leads us to hypothesize that regulation of vacuole size is a general mechanism to regulate cell expansion and that other players besides NET4 must participate in regulating the vacuole–cytoskeleton interface.

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The phytohormone auxin is a vital growth regulator with diametrical effects on roots and shoots. Increased abundance of root tissue leads to inhibition of cellular elongation.[1] For root epidermal cells of *Arabidopsis thaliana*, it has been shown that growth inhibition by auxin is a result of changed vacuolar morphology.[2] Auxin induced more constricted, tubular vacuoles, which occupied less cellular space than usual.[3] This led to the conclusion that vacuoles have a space-filling function during growth. Therefore, it was hypothesized that this mechanism allows rapid cell elongation with relatively little energy investment.[3–7]

The observed auxin-induced vacuolar constrictions and the resulting space constrains were shown to be dependent on the actin cytoskeleton.[3] To shed light on the cytoskeleton–vacuole connection, members of the actin-binding Networked (NET) 4 protein family,[8] which are localized at the tonoplast, were investigated.[5] While a *net4a net4b* double mutant showed only a weak phenotype, overexpression of NET4A in a 35S::NET4A-GFP line (NET4A-GFP^{OE}) led to more spherical and compact vacuoles, which consequently also occupied less cellular space.[5] Despite morphological differences of vacuoles in NET4A-GFP^{OE} and auxin-treated vacuoles in Col-0 wild type, reduced occupancy in both cases was accompanied by reduced cell size and root length.[2,5] Therefore, it seems plausible that reducing the vacuolar occupancy of the cell, regardless of the underlying mechanism, inhibits cellular elongation and eventually root growth in general.

To analyze this further, we examined the vacuolar occupancy of NET4A-GFP^{OE} and *net4a net4b* compared to Col-0 wild type upon treatment with the synthetic auxin naphthalene acetic acid (NAA). To this end, the vacuolar lumen of *Arabidopsis* root epidermal cells within the late meristematic zone was stained with BCECF (2′,7′-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein) and the corresponding cell walls with propidium iodide (PI) as described previously in Scheuring et al.[9] Based on 3D modeling of vacuoles and cells,[4] vacuolar occupancy was quantified. While vacuoles of all tested lines showed tubular constrictions, vacuoles of NET4A-GFP^{OE} remained spherical and more compact (Figure 1a–c). This is in accordance with previous findings, showing no differences in the auxin-induced decrease in the vacuolar morphology index, as a measure for the extent of vacuolar constrictions.[5] On the other hand, it led to an additional increase in compactness of the already more compact vacuoles in NET4A-GFP^{OE}.[5] It has been shown that in general, auxin-induced constrictions lead to a reduced vacuolar occupancy in root meristematic cells.[3] This finding in combination with the observed higher compactness of NET4A-GFP^{OE} vacuoles tempted us to expect a stronger reduction of vacuolar occupancy when the overexpressor is treated with NAA. However, despite the described effects on vacuolar morphology and compactness, the occupancy of NET4A-GFP^{OE} was higher than that of the control (Figure 1d). Consequently, quantification of occupancy upon auxin treatment relative to control conditions revealed a significantly lower decrease for NET4A-GFP^{OE} than for the control (Figure 1e), indicating a partial resistance. In contrast, quantification of the occupancy as well as the relative change of occupancy upon NAA treatment for the *net4a net4b* double

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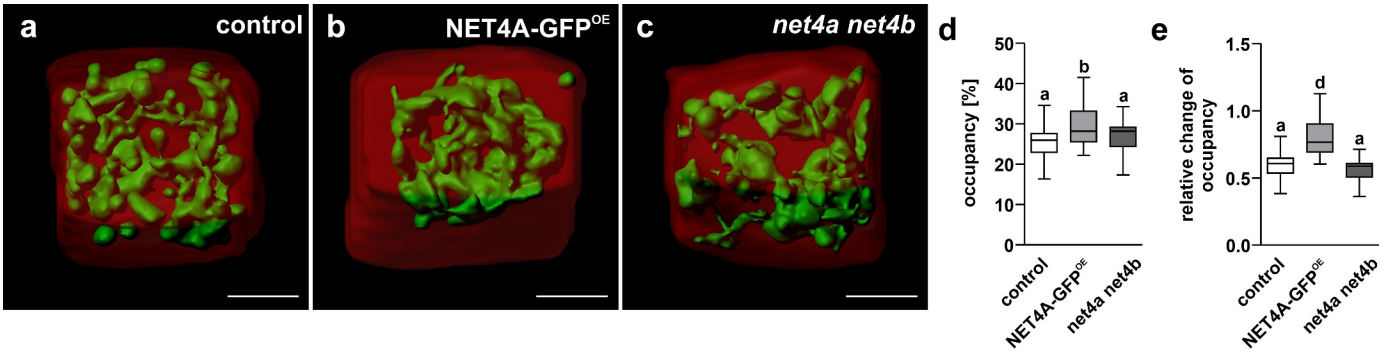

Figure 1. Only vacuoles of NET4A-GFP^{OE} show partial resistance to auxin treatment. (a–c) Three-dimensional modeling (using Imaris software) of PI-stained cell walls (red) and BCECF-stained vacuoles (green) of late meristematic root cells from Columbia-0 control (n = 27), NET4A-GFP^{OE} (n = 16) and *net4a net4b* (n = 14) upon auxin treatment (200 nM NAA, 20 h). (d) Quantification of the vacuolar occupancy of the cell (occupancy) upon auxin treatment. (e) Change in vacuolar occupancy upon auxin treatment relative to untreated conditions. Box limits of boxplots display 25th percentile and 75th percentile, horizontal line depicts median. Whiskers represent minimum to maximum values. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test, p-value b: $p < .05$, d: $p < .001$ compared to a. Scale bars: 5 µm.

mutant showed no significant difference in comparison to the wild-type control (Figure 1d,e). These findings indicate that neither NET4A nor NET4B are essential for auxin-mediated vacuolar constrictions. Instead, it might be assumed that NET4A-mediated increase in vacuolar compactness relies on an additional pathway, which is only indirectly affected by auxin. Hence, the observed partial auxin resistance of NET4A-GFP^{OE} might be explained by already smaller and more compact vacuoles, which only allow constrictions to a certain extent.

Since all members of the NET superfamily possess actin-binding domains and are associated with different membranes, it is also conceivable that a lacking phenotype of *net4a net4b* is due to functional redundancy by other NETs. To test for possible transcriptional upregulation of other NETs in the *net4a net4b* mutant background, we performed real-time quantitative PCR[10] for all family members of NET1, NET2 and NET3. Transcript levels of 7-d-old Arabidopsis seedlings in the double mutant background were compared to control (Col-0) levels (Figure 2). While for *NET2A-C* and *NET3B* expression was not detectable, all other *NETs* were not upregulated, giving no indication of a compensatory effect in the *net4a net4b* double mutant.

In general, the molecular function of NET4 is far from being understood. Actin-binding capacity and localization at the tonoplast suggest a regulatory function of the cytoskeleton at the vacuole. In this scenario, the more spherical and compact vacuoles of NET4A-GFP^{OE} would be followed by changes of the actin cytoskeleton. Recently, it has been shown that NET3C not only binds to actin and the plasma membrane (PM) but also forms complexes with other proteins – vesicle-associated protein 27 (VAP27), kinesin light chain-related protein 1 (KLCR1), IQ67 domain 2 (IQD2) – at contact sites of the PM and the endoplasmic reticulum (ER). Notably, these complexes bridge the actin and microtubule network and seem to directly affect the morphology of the ER.[11,12] It is conceivable that similar complexes including NET4 might be formed at specific tonoplast sites.

Functional reasons to have more compact vacuoles might be manifold. Besides slowing down growth, for instance, as reaction to certain stresses, preventing the tonoplast from coming in direct contact with the PM could also have an influence on cytoplasmic streaming. Interestingly, it has been demonstrated that accelerating or decelerating cytoplasmic streaming velocity correlated with increased or reduced cell size and overall plant growth, respectively.[13] Hence, one could even assume

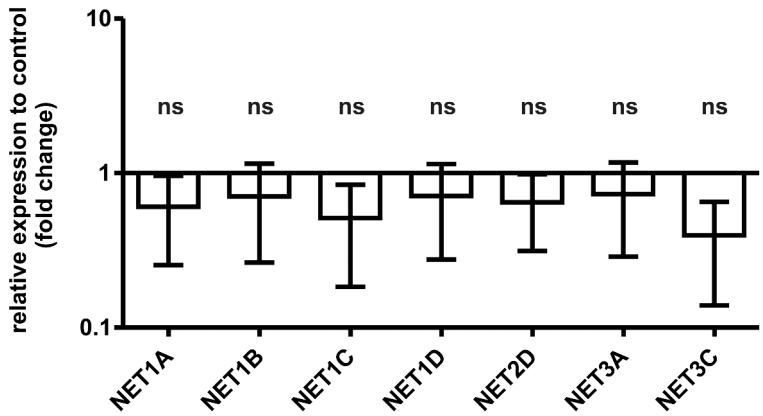

Figure 2. No compensatory upregulation on transcript level is detectable in the *net4a net4b* double mutant. Expression levels of NET1-3 in *net4a net4b* are presented as the mean fold change to the expression levels in Columbia-0 wild-type control on a logarithmic scale. Experiment was performed thrice with at least two technical replicates. Error bars represent standard error of the mean. Student's t-test was used for statistical analysis, ns: not significant.

some kind of feedback regulation. Apart from that, the proximity between the tonoplast and the nucleus (especially in NET4A-GFP^{OE}) could also indicate some connection to nucleus positioning. It has been reported that positioning of the nucleus in root hairs is regulated by modulation of the filamentous actin organization.[14,15]

However, the lack of a strong phenotype in the *net4a net4b* double mutant implies that a higher molecular complexity provides redundancy for tethering the actin cytoskeleton to the tonoplast. The finding that NET4 seems not to be essential for mediating auxin-dependent changes in the vacuolar morphology[5] makes it tempting to assume that there must be additional molecular players. It will be an exciting future task to identify these yet unknown players and to decipher molecular processes at the vacuole–cytoskeleton interface.

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Disclosure of potential conflicts of interest

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