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SHORT COMMUNICATION

A proposed role for endomembrane trafficking processes in regulating tonoplast content and vacuole dynamics under ammonium stress conditions in Arabidopsis root cells

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ABSTRACT

Ammonium (NH_4^+) stress has multiple effects on plant physiology, therefore, plant responses are complex, and multiple mechanisms are involved in NH_4^+ sensitivity and tolerance in plants. Root growth inhibition is an important quantitative readout of the effects of NH_4^+ stress on plant physiology, and cell elongation appear as the principal growth inhibition target. We recently proposed autophagy as a relevant physiological mechanisms underlying NH_4^+ sensitivity response in Arabidopsis. In a brief overview, the impaired macro-autophagic flux observed under NH_4^+ stress conditions has a detrimental impact on the cellular energetic balance, and therefore on the energy-demanding plant growth. In contrast to its inhibitory effect on the autophagosomes flux to vacuole, NH_4^+ toxicity induced a micro-autophagy-like process. Consistent with the reduced membrane flux to the vacuole related to macro-autophagy inhibition and the increased tonoplast degradation due to enhanced micro-autophagy, the vacuoles of the root cells of the NH_4^+-stressed plants showed lower tonoplast content and a decreased perimeter/area ratio. As the endosome-to-vacuole trafficking is another important process that contributes to membrane flux toward the vacuole, we evaluated the effects of NH_4^+ stress on this process. This allows us to propose that autophagy could contribute to vacuole development as well as possible avenues to follow for future studies.

Ammonium (NH_4^+) is a major inorganic nitrogen form that plants can take from soils. However, meanwhile low external NH_4^+ supply promotes plant growth, high NH_4^+ concentration causes toxicity.[1,2] NH_4^+ toxicity impacts on multiple cellular events; therefore plant responses to NH_4^+ stress are complex.[1,2] One of the most visible phenotype of the NH_4^+ toxicity syndrome is root growth inhibition.[1,2] Given the pleiotropic effects of NH_4^+ toxicity on plant physiology, several hypotheses have been advanced to explain NH_4^+ toxicity syndrome.[1,2] One of the effects of NH_4^+ toxicity on plant growth is associated with carbohydrate consumption and the energy costs required to cope with NH_4^+ stress.[3] Consequently, NH_4^+ toxicity is augmented when light energy or carbohydrates are limiting, and, as we recently reported, when autophagy, a process related to the provision of energy, is impaired.[4] In this short communication, we focused the discussion on the effects of NH_4^+ toxicity on endomembrane trafficking pathways, autophagy and endocytosis, and their impact on vacuole dynamics and cell elongation.

According to several studies, different key cellular processes underlying cell elongation are affected under NH_4^+ stress conditions.[5–12] One important factor controlling cell elongation is the cell wall, a rigid structure that needs to be modified for allowing cell growth,[13] and which become more rigid under NH_4^+ conditions.[10] Furthermore, aligned with the cell wall status, it has been reported the relevance of controlling the dynamics and volume of vacuoles for plant cell growth, thus playing a role in cell size determination.[14–16] Under this scenario, we recently reported that the late steps of the macro-autophagic flux were impaired under NH_4^+ conditions, resulting in autophagosomes accumulation in the cytosol, while micro-autophagic activity was induced in root cells (Figure 1a, b, e, f). We proposed that the differential activity of these two processes under severe NH_4^+ conditions may impact vacuole dynamics and tonoplast content in root cells (Figure 2).[4]

Macro-autophagy is a specialized endomembrane trafficking process through which different cargos are recognized, enclosed and transported by the autophagosomes, unique double membrane vesicles, to the vacuole.[17] Previous studies have proposed that membrane flux from macro-autophagy may contribute to the biogenesis and enlargement of vacuoles.[18–21] Through micro-autophagy, about which comparatively little is known in plants, cytoplasmic constituents are incorporated into the vacuolar lumen directly by vacuole-membrane invaginations.[22] It has been suggested in yeast that micro-
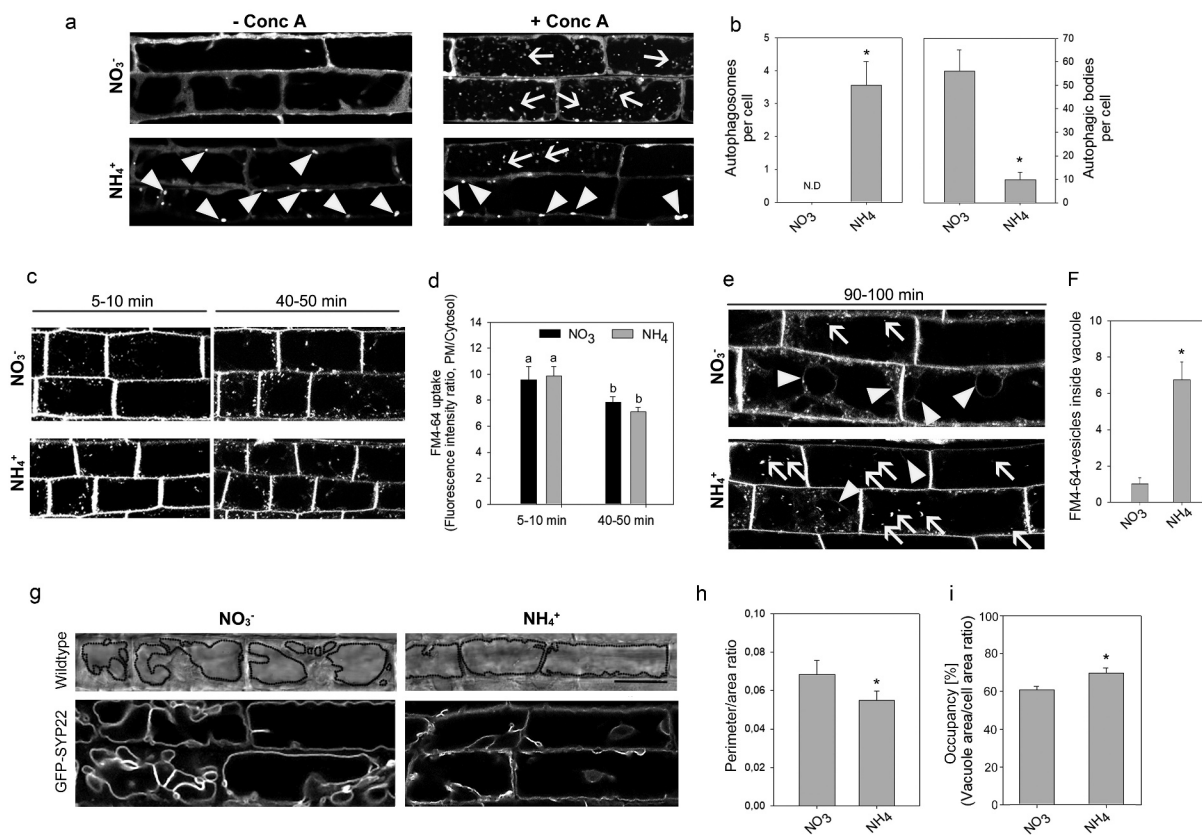
Figure 1. Endomembrane-to-vacuole trafficking in root cells of 7 days-old seedlings grown under NO_3^- and NH_4^+ conditions. (a–b) NH_4^+ toxicity affects the autophagic flux to the vacuole. Plants expressing GFP-ATG8a were grown under NO_3^- and NH_4^+ conditions for 7 days, and autophagosomes (-Conc A) and autophagic bodies (+ Conc A) were visualized by confocal microscopy (a). Arrowheads indicate autophagosomes, arrows indicate autophagic bodies. The average of autophagosome (*left*) and autophagic body (*right*) number per cell was calculated from single-plane confocal images, per plant (b). Five plants were analyzed (and more than four cells per plant). Asterisks indicate significant differences ($p < .05$, DGC test). N.D: non-detected. (c–f) The endosome-to-vacuole trafficking was analyzed in seedlings grown with the indicated N sources for 7 days, incubated for 5 min (c–d) or 15 min (e–f) with 5 µg ml^{-1} FM4-64, and then observed by confocal microscopy after (c–d) 5–10 min, 40–50 min and (e–f) 90–100 min. (d) FM4-64 uptake was estimated as the fluorescence ratio between plasma membrane (PM) and cytosol using the software ImageJ. Six plants were analyzed (and more than five cells per plant). Different letters indicate significant differences ($p < .05$, DGC test). (e) Arrowheads indicate FM4-64 tonoplast staining. Arrows indicate FM4-64 vesicles inside the vacuole. (f) The average number of FM4-64 vesicles per cell was calculated from single-plane confocal images for each plant. At least five plants were analyzed (and more than five cells per plant). Asterisks indicate significant differences ($p < .05$, DGC test). G-l) Vacuole morphology in root cells of 7 days-old seedlings grown under the indicated N sources. (g) Representative images of the vacuole morphology in wild-type (*upper panel*) and GFP-SYP22 transgenic plants (*lower panel*). (h) Vacuolar perimeter to area ratio, and (i) vacuole area to cell area ratio (occupancy) were calculated using ImageJ software. Five plants were analyzed (and more than five cells per plant). Asterisks indicate significant differences ($p < .05$, DGC test).
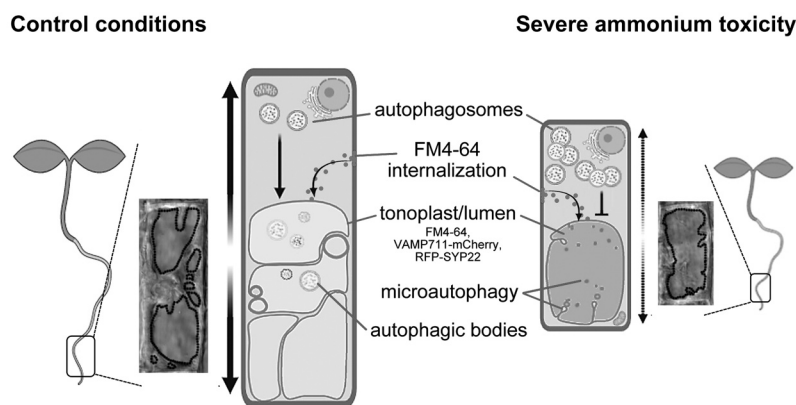
Figure 2. Model depicting the effects of NH_4^+ toxicity on endomembrane trafficking processes and their possible effects on tonoplast content and vacuole morphology. Under severe NH_4^+ stress conditions macro-autophagic flux is slowed and autophagosomes accumulate in cytosol, while the internalization and degradation of the tonoplast increases through a micro-autophagy-like process. The reduced membrane flux to the vacuole, apparently not so much from endosomes but from autophagosomes, together with the increased tonoplast degradation may impact on tonoplast content, and hence affect vacuole dynamics. Further studies are needed to unravel whether macro-autophagy and micro-autophagy have a role in the development and dynamics of vacuoles in plants, and their impact on cell elongation.

autophagy may function in regulating tonoplast size in response to the membrane influx from the macro-autophagic flux.[23–25] However, we recently found that NH_4^+ stress increases micro-autophagic activity while impairing macro-autophagic flux in Arabidopsis roots, thus creating a global imbalance in the membrane flow to/from the tonoplast (Figure 1a, b, e, f).[4] Nevertheless, the proposal of micro-autophagy regulating tonoplast size assumes that this process is functionally interconnected with other endomembrane transport processes including not only macro-autophagy, but also endosome-to-vacuole trafficking pathways. In this sense, it has been postulated the role of endocytic pathways in vacuole biogenesis and dynamic (revised by[26,27]). Therefore, given this background, we monitored the endocytic process toward the vacuole in the root cells of plants grown under NO_3^- (KNO_3 5 mM) and NH_4^+ (NH_4-succinate 2.5 mM) conditions by staining the plasma membrane with the lipophilic dye FM4-64 (Figure 1c–f). At early time points after FM4-64 staining (5–10 and 40–50 min), no obvious differences were observed in the internalization of the probe between the treatments (Figure 1c, d) suggesting that the early steps of endocytosis were not affected in root cells under NH_4^+ stress conditions. After 90–100 min, FM4-64-tonoplast staining began to be observed in root cells, being more evident in plants grown under NO_3^- conditions (arrowheads in Figure 1e). Could late stages of endosome-to-vacuole trafficking be affected under conditions of NH_4^+ toxicity? Simultaneously with the beginning of tonoplast staining, FM4-64-vesicles appeared inside the vacuole in plants grown under NH_4^+ conditions (Figure 1e, f), suggesting that the slight delay in tonoplast staining could be due to a rapid clearance of FM4-64 from the tonoplast by an induced micro-autophagy-like process (arrows in Figure 1e) rather than defects in the endosome-to-vacuole trafficking pathways. Nevertheless, we should not exclude possible NH_4^+ toxicity effects on vesicle trafficking pathways, as suggested by previous reports.[4,10,28]

Vacuole morphology in growth competent root cells was affected under NH_4^+ stress conditions (Figure 1g-i). The vacuolar perimeter to area ratio was lower in root cells of the elongation zone in plants grown under NH_4^+ conditions, showing the vacuoles a less convoluted structure compared to those observed in plants grown under NO_3^- conditions (Figure 1g,h). Under this scenario, we proposed that the global imbalance created in the membrane flow to/from the vacuole, principally due to the differential activation of the autophagic processes (macro-autophagy and micro-autophagy), impacts on vacuole development and tonoplast content (Figure 2). During cell growth, the increase in vacuolar occupancy supports the expansion of root cell.[16] In order to estimate the cell volume occupied by vacuoles, the vacuole area to cell area ratio was calculated (Figure 1i). Interestingly, although root cell size was smaller in plants grown under NH_4^+ conditions compared to NO_3^- conditions, the vacuolar occupancy of the cell was higher in the former (Figure 1g, i). The usage of tonoplast reservoirs (through changes in the perimeter/area ratio) that support vacuole expansion and increase the vacuolar occupancy,[16] might contribute to root cell elongation under NH_4^+ stress conditions because it functions independently of new membrane synthesis and influx to

the vacuole. Overall, vacuoles with reduced tonoplast reservoirs (ultimately less tonoplast content) will have a lesser capacity to increase in size through dynamic morphological changes in response to various growth and environmental signals. Besides, as it was mentioned above, cell elongation required the spatial and temporal coordination between the increased vacuolar size and cell wall loosening, which, in turn, is affected under NH_4^+ stress conditions, limiting cell expansion.[10] Although new evidence is provided supporting the notion that autophagy (macro-autophagy and micro-autophagy) may have effects on vacuolar enlargement,[4,19–21,29] further investigations are needed to unravel the autophagic role in vacuole development, and its impact on vacuole dynamics and cell elongation in plants.

Disclosure statement

No potential conflicts of interest were disclosed.

Data availability statement:

All relevant data can be found within the manuscript

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