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Activation of the plasma membrane H+-ATPase by acid stress

Antibodies as a tool to follow the phosphorylation status of the penultimate activating Thr

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Tight regulation of the plasma membrane proton pump ATPase (H+-ATPase) is necessary for controlling the membrane potential that energizes secondary transporters. This regulation relies on the phosphorylation of the H+-ATPase penultimate residue, a theonine, and the subsequent binding of regulatory 14-3-3 proteins, which results in enzyme activation. Using phospho-specific antibodies directed against the phosphorylable Thr of either PMA2 (Plasma membrane H+-ATPase from *N. plumbaginifolia*) or PMA4, we showed that the kinetics and extent of phosphorylation differ between both isoforms according to the growth or environmental conditions like cold stress.[1] Here, we used phospho-specific antibodies to follow PMA2 Thr phosphorylation upon acidification of the cytosol by incubating *N. tabacum* BY2 cells with four different weak organic acids. Increased PMA2 phosphorylation was observed for three of them, thus highlighting the role of the H+-ATPase in cell pH homeostasis.

The H+-ATPase is a major enzyme of the plant plasma membrane. This P-type ATPase couples ATP hydrolysis with proton transport out of the cell and establishes pH and potential gradients across the plasma membrane, thereby activating secondary transporters. At the physiological level, this enzyme is implicated in diverse roles, such as cytosolic pH regulation, cell elongation or stomata aperture.[2,3] H+-ATPase consists of ten membrane spanning regions and four cytosolic domains, among which the auto-inhibitory C-terminal region. The activation mechanism of the enzyme is well known and involves phosphorylation of its penultimate residue, a threonine, by an as yet unidentified protein kinase; phosphorylation in turn leads to the binding of regulatory 14-3-3 protein dimers and to the formation of an activated complex consisting of six H+-ATPases and six 14-3-3 proteins.[4-7]

Additional conserved phosphorylation sites in the enzyme C-terminal region have been shown to positively or negatively contribute to the enzyme regulation.[8-10] More sites have been discovered by large-scale phospho-proteomics, but have not been studied to date.[10,11] Most of these additional phosphorylated residues are located in the enzyme C-terminal auto-inhibitory domain. This domain contains two to three inhibitory regions and a 14-3-3 binding region, partially superimposed with an inhibitory region.[12,13] All these recent data suggest that the activity of H+-ATPase is finely tuned. However, the complexity of this regulation makes it difficult at the present stage to propose a comprehensive view.

To follow and compare the activation status of two H+-ATPase isoforms belonging to different subfamilies, antibodies were designed for specifically recognizing the phosphorylated form of the penultimate Thr of either PMA2 (Plasma membrane H+-ATPase from *N. plumbaginifolia*) or PMA4, two broadly expressed isoforms belonging to subfamily I and II, respectively. This allowed us to find, for example, that PMA2, as opposed to PMA4, is strongly dephosphorylated upon

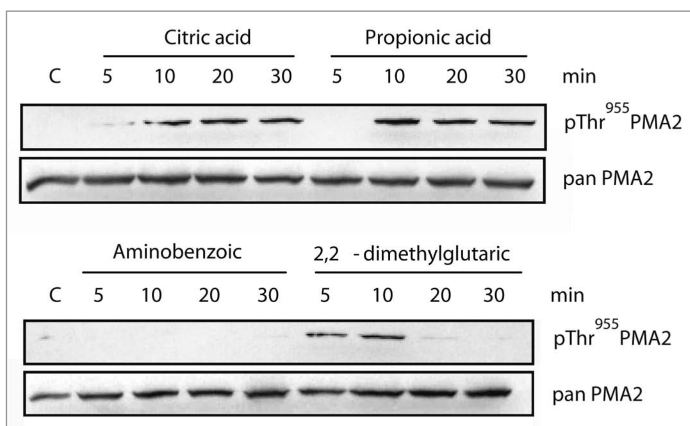

Figure 1. Effect of various weak acids on the phosphorylation of the PMA2 penultimate Thr residue of *N. tabacum* suspension cells. A 3-day old *N. tabacum* BY2 cell culture was treated with 1/10th volume of 50 mM of either aminobenzoic acid, 2,2-dimethylglutaric acid, citric acid or propionic acid, dissolved in the culture medium and brought beforehand to the same pH as the culture. After the indicated periods of time, cells were collected and a microsomal fraction was isolated and analyzed by western blotting using antibodies pThr955PMA2 recognizing the PMA2 penultimate activating Thr[1] (upper) and pan PMA2 recognizing a short sequence specific for PMA2,[14] (lower). C, untreated cells.

cold stress. Both isoforms are strongly activated, upon subculturing *N. tabacum* BY2 suspension cells into a new media.[1] However, they underwent dephosphorylation at different rates as the cell culture proceeded. These data showed the usefulness of these antibodies for determining the regulation of specific H^+-ATPase isoforms and better understanding their physiological roles.

The primary function of the plasma membrane H^+-ATPase is to transfer protons outside the cell. H^+-ATPase is therefore considered as a possible regulator of the cytosolic pH homeostasis, for instance by preventing internal acidification. However, few data so far support this role for H^+-ATPase. We addressed this point by adding to a *N. tabacum* BY2 cell culture weak organic acids, which are expected to permeate the membrane as a protonated form and dissociate once inside, resulting in cytosol acidification. This is expected to activate the plasma membrane H^+-ATPase and so remove the proton excess out off the cell. A *N. tabacum* BY2 cell culture was treated with 5 mM of either citric acid, aminobenzoic acid, 2,2-dimethylglutaric acid, or propionic acid (**Fig. 1**). After several periods of time, a microsomal fraction was isolated and analyzed by western blotting. Among the four weak acids tested, propionic acid and citric acid induced strong and stable increase of PMA2 penultimate Thr phosphorylation. 2,2-dimethylglutaric acid induced a temporary increase of phosphorylation while aminobenzoic acid had no effect. The different responses might be explained either by different diffusion rates of the organic acids across the plasma membrane or by their possible toxicity. In addition, homeostasis of the intracellular pH results from the activity of several different enzymatic systems such as vacuolar H^+-ATPase and H^+-pyrophosphatase. Therefore it is also possible that, depending on the rate and/or extent of cytosol acidification, different responses are activated.

This data supports the role H^+-ATPase in pH homeostasis and highlights the strong potential of using phospho-specific antibodies to follow enzyme activation in the plant according to different environmental conditions. In addition, one should also take advantage of them as a tool for in vitro phosphorylation tests using different subcellular fractions of *N. tabacum* BY2 cells. This approach might lead to the isolation of the kinase and phosphatase involved in the modification of the penultimate Thr residue. Indeed, these enzymes are still undiscovered in spite of the fact that phosphorylation of this residue has been demonstrated more than a decade ago.

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