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Endomembrane Ca^{2+}-ATPases play a significant role in virus-induced adaptation to oxidative stress

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Although the role of Ca^{2+} influx channels in oxidative stress signaling and cross-tolerance in plants is well established, little is known about the role of active Ca^{2+} efflux systems in this process. In our recent paper,[17] we reported *Potato Virus X* (PVX)-induced acquired resistance to oxidative stress in *Nicotiana benthamiana* and showed the critical role of plasma membrane Ca^{2+}/H^{+} exchangers in this process. The current study continues this research. Using biochemical and electrophysiological approaches, we reveal that both endomembrane P_{2A} and P_{2B} Ca^{2+}-ATPases play significant roles in adaptive responses to oxidative stress by removing excessive Ca^{2+} from the cytosol, and that their functional expression is significantly altered in PVX-inoculated plants. These findings highlight the crucial role of Ca^{2+} efflux systems in acquired tolerance to oxidative stress and open up prospects for practical applications in agriculture, after in-depth comprehension of the fundamental mechanisms involved in common responses to environmental factors at the genomic, cellular and organismal levels.

Key words: cytosolic calcium, reactive oxygen species, cross-tolerance, calcium pump

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The phenomenon of cross-tolerance to a variety of biotic and abiotic stresses is well-known.[1,2] Some of the demonstrated examples include the correlation between oxidative stress tolerance and pathogen resistance.[3-5] At the mechanistic level, changes in cytosolic Ca^{2+} levels $[Ca^{2+}]_{cyt}$, have long been implicated as a quintessential component of this process.[6] The rise in $[Ca^{2+}]_{cyt}$ is proven to be essential for the development of the oxidative burst required for triggering the activation of several plant defense reactions.[7,8] The observed elevation in H_2O_2 level is believed to result from Ca^{2+}-dependent activation of the NADPH oxidase,[8] which then causes a further increase in $[Ca^{2+}]_{cyt}$ via a positive feedback mechanism. This process is further accomplished by defense gene activation, phytoalexin synthesis and eventual cell death.[9] Downstream from the stimulus-induced $[Ca^{2+}]_{cyt}$ elevation, cells possess an array of proteins that can respond to a message. Such proteins include calmodulin (CaM),[10] Ca^{2+}-dependent protein kinases[11] and CaM binding proteins.[12] Of note is that when Ca^{2+} channels are blocked, biosynthesis of ROS is prevented.[13]

While the role of Ca^{2+} influx channels in oxidative stress signaling and cross-tolerance in plants is well established, little is known about the involvement of active Ca^{2+} efflux systems in this process. In contrast, in animal systems the essential role of re-establishing $[Ca^{2+}]_{cyt}$ to resting levels is widely reported. A sustained increase in $[Ca^{2+}]_{cyt}$ in the alveolar macrophage is thought to be the consequence of membrane Ca^{2+}-ATPase dysfunction.[14] In endothelial cells, inhibition of the Ca^{2+}/Na^{+} electroneutral exchanger of the mitochondria was named as one of the reasons for $[Ca^{2+}]_{cyt}$ increases.[15] A significant loss of the plasma membrane Ca^{2+}-ATPase

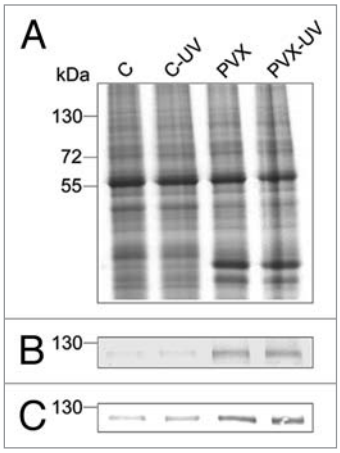

Figure 1. Expression of P_{2B} Ca^{2+} in purified microsomal fractions from tobacco leaves. Measurements were undertaken C = mock controls; C-UV = mock controls treated with UV-light; PVX = PVX infected plants; PVX-UV = PVX inoculated plants treated with UV-light. (A) Coomassie Brilliant Blue-stained gel; (B) Protein blot immunostained with a non isoform-specific polyclonal antibody for P_{2B} Ca^{2+}-ATPases; (C) CaM overlay assay.

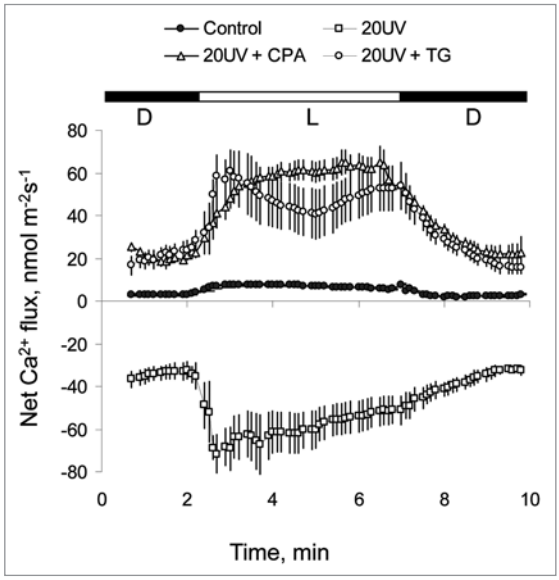

Figure 2. Effect of known Ca^{2+}-ATPase blockers on light-induced Ca^{2+} flux kinetics after 20 min of UV-C treatment. Leaf mesophyll segments were pre-treated in either 5 μM TG (thapsigargin) or 50 μM CPA (cyclopiazonic acid) for 1–1.5 h prior to exposure to UV-C light. Net Ca^{2+} fluxes were measured 2 h after the end of UV treatment. These were compared with two controls: (1) no pre-treatment/no UV exposure (closed circles) and (2) no pre-treatment/20 min UV exposure (open squares). Mean ± SE (n = 4 to 7).

(PMCA) activity was reported in brain synapses in response to oxidative stress,[16] suggesting that PMCA may be a downstream target of oxidative stress.

In our recently published paper[17] we reported the phenomenon of *Potato Virus X* (PVX)-induced acquired resistance to oxidative stress in *Nicotiana benthamiana* plants and showed the critical role of plasma membrane Ca^{2+}/H^{+} exchangers in this process. Nonetheless, questions remain, is this transporter the only active Ca^{2+} efflux system involved in this process?

In addition to Ca^{2+}/H^{+} exchangers, active Ca^{2+} extrusion could also be achieved by Ca^{2+}-ATPases. Two major types of Ca^{2+}-ATPases that differ substantially in their pharmacology and sensitivity to CaM are known.[18] Type P_{2A} pumps (also called ER-type or ECA[19,20]) are predominantly ER-localized,[19] although they are also present at other endomembranes (e.g., tonoplast and Golgi). Four members of this group have been identified in the Arabidopsis genome (named AtECAs 1 to 4).[18,21] These pumps lack an N-terminal autoregulatory domain, are insensitive to CaM and suppressed by cyclopropiazonic acid (CPA).[19] P_{2B} (or ACA) pumps contain an autoinhibitory N-terminal domain that possesses a binding site for Ca^{2+}-CaM.[18] Ten members are known in Arabidopsis (termed AtACA1, 2, 4 and 7 to 13).[21] Plant P_{2B} pumps are located at the plasma membrane[20] as well as in inner membranes such as tonoplast (e.g., ACA4), ER (e.g., ACA2) and plastids.[18,19] These pumps probably constitute the basis for precise cytosolic Ca^{2+} regulation; as the Ca^{2+} concentration increases, CaM is activated and binds to the autoinhibitory domain of the Ca^{2+} pump. This results in the activation of the pump.

In our recent study,[17] we found no significant difference between the purified plasma membranes fractions isolated from control and UV-treated tobacco plants (with or without PVX inoculation) either in the Ca^{2+}-ATPase activity or in the Ca^{2+}-ATPase expression level and its ability to bind CaM. This suggests that the plasma membrane P_{2B} type pumps (the only pump type known to be expressed at the plasma membrane) play no major role in removing excess Ca^{2+} from the cytosol under oxidative stress conditions. This led to an obvious question: what about endomembrane Ca^{2+}-ATPases?

To address this issue, microsomal membrane fractions were isolated from tobacco leaves in a manner previously described for plasma membrane fractions[17] (**Fig. 1A**). Western blot and CaM overlay assays were then made to investigate the role of endomembrane P_{2B} Ca^{2+}-ATPases in our reported phenomena of acquired resistance. The results show that the expression of the P_{2B} Ca^{2+} pumps in PVX-inoculated plants is significantly higher than in control plants (**Fig. 1B**), correlating well with the CaM overlay assay (**Fig. 1C**). As no difference was observed for the P_{2B} Ca^{2+}-ATPase expression levels in the plasma membranes,[17] the observed difference in the microsomal fractions of PVX-infected plants must be due to an

increased expression of endomembrane P_{2B} Ca^{2+}-ATPases. Given the fact that Ca^{2+} pumps have a high affinity for calcium, the observed increase in endomembrane P_{2B}-type Ca^{2+}-ATPases expression in PVX-inoculated plants may be advantageous for more efficient Ca^{2+} removal from the cytosol into internal organelles.

To decipher the possible role of P_{2A} Ca^{2+}-ATPases in acquired resistance, a series of electrophysiological experiments were conducted using inhibitors of P_{2A}-type Ca^{2+}-ATPases, such as thapsigargin (TG) [22] and cyclopiazonic acid (CPA).[23] Ion-selective Ca^{2+} microelectrodes were prepared as described elsewhere in reference 24 and 25, and net Ca^{2+} fluxes were measured from tobacco mesophyll tissue following previously described protocols.[17] Leaf pre-treatment for 2 h in either of these inhibitors dramatically suppressed the net Ca^{2+} efflux measured from tobacco mesophyll cells 2 h after UV light exposure (**Fig. 2**). Given the specificity of TG and CPA inhibitors for P_{2A}-type Ca^{2+}-ATPases, these results strongly support a hypothesis that both endomembrane P_{2A} and P_{2B} Ca^{2+}-ATPases play significant roles in plant adaptive responses to oxidative stress. This is achieved by removing excess Ca^{2+} from the cytosol.

Combining these results with our previously reported observations in reference 17, the following model is proposed (**Fig. 3**). Oxidative stress (such as UV) causes increased ROS production in leaf chloroplasts, leading to the elevated $[Ca^{2+}]_{cyt}$. Several Ca^{2+} efflux systems are involved in restoring basal cytosolic Ca^{2+} levels. Two of these, the plasma membrane Ca^{2+}/H^+ exchanger[17] and endomembrane P_{2A} and P_{2B} Ca^{2+}-ATPases (as reported in this study) are upregulated in PVX inoculated plants and contribute to the improved tolerance to oxidative stress. Overall, these findings highlight the potential role of Ca^{2+} efflux systems in virus-induced tolerance to oxidative stress in plants. This is consistent with our previous reports on the important role of Ca^{2+} efflux systems in biotic stress tolerance[26] and brings forth possibilities for genetic engineering of more tolerant plants by targeting expression and regulation of active Ca^{2+} efflux systems at either the plasma or endomembranes.

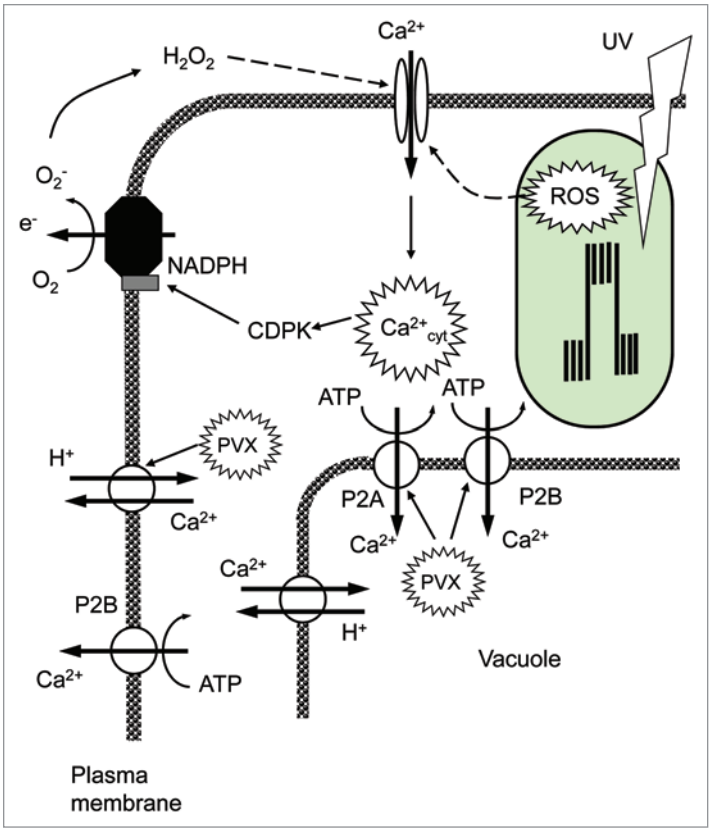

Figure 3. The proposed model of oxidative stress signaling and the role of Ca^{2+}-efflux systems in acquired resistance and plant adaptation to oxidative stress.

Overall, a better adaptation of virus-infected plants to a short wave UV irradiation as compared to uninfected controls may suggest that infection triggers common defense mechanisms that could be efficient against secondary unrelated stresses. This observation may lead to the development of novel strategies to protect plants against complex environmental stress conditions.

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