Evaluation of Ca content and distribution in leaf of plants treated with different Ca-based formulates

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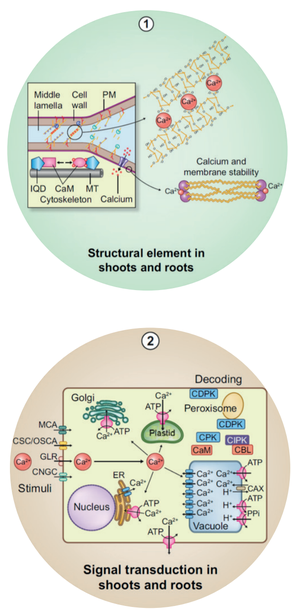
Calcium ion (Ca^{2+}) represents one of the essential macronutrient for plants. Most of the functions of Ca^{2+} as a structural or regulatory component of macromolecules are related to its capacity for coordination, by which it provides stable but reversible molecular linkages. In contrast to other macronutrients, a high proportion of the total Ca^{2+} in plant tissues is often located in cell walls (apoplasm). A large amount of Ca^{2+} is often sequestered into vacuoles, whereas its concentration in the cytosol is kept at very low levels (in a range of 100-200 nM concentrations) to prevent toxic effects.

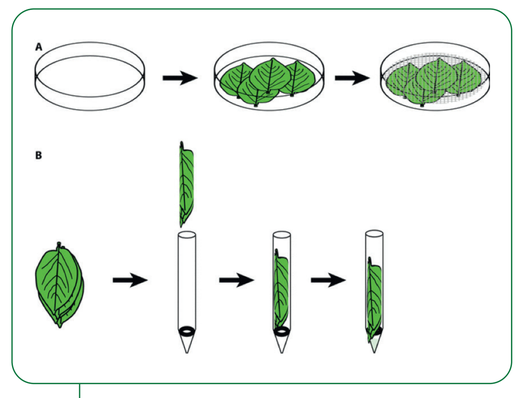


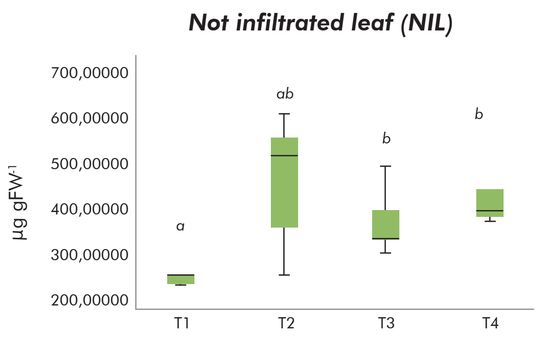
The Ca role in plant cells

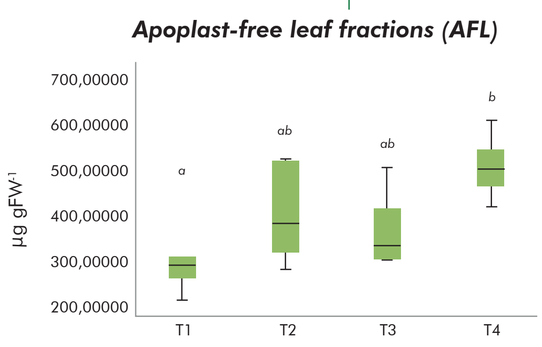
This work aimed to determine the Ca content and distribution in the leaf of plants treated with three different Ca-based formulates (GHI_789 (T2), GHI_049 (T3), $CaCl_2$, (T4)). Such investigation has been performed on lettuce and Arabidopsis leaves using **leaf tissue fractionation and microscope-based fluorescent approaches.**

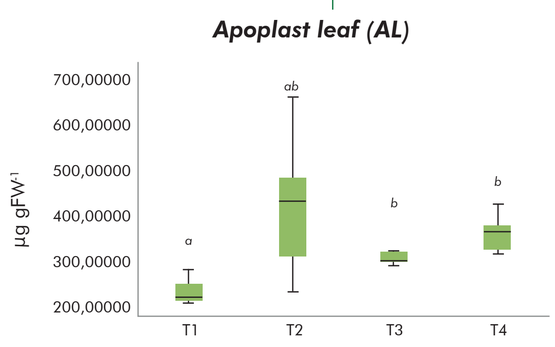
Freshly excised lettuce leaves were rinsed by submersion in distilled water to remove leaf surface contaminants. Portion of leaf from lettuce plants were infiltrated with ultra-pure water using a 60 ml syringe. Carefully, infiltrated leaves were rolled and inserted into a 60 ml syringe and the syringe was filled with distilled water.

Ejection of any air within the syringe were performed by lowering the plunger to approximately the 40 ml mark. Once the leaves were fully infiltrated, as seen by the darkened colour of the infiltrated areas, they were removed from the syringe, and the weight were measured after removing the residual water on the leaf surface. The leaves were then placed on a piece of 4 inch (10 cm) wide Parafilm. Using a 5 ml pipette tip (or a similar sized object), the leaves were rolled up the within the Parafilm. The rolled up leaves were inserted with pipette tip into a 20 ml syringe. Apoplast leaf (AL) and the relative Apoplast-free leaf fractions were obtained after centrifugation for 10 min at 1,000 x g in a swinging bucket rotor at 4 °C.

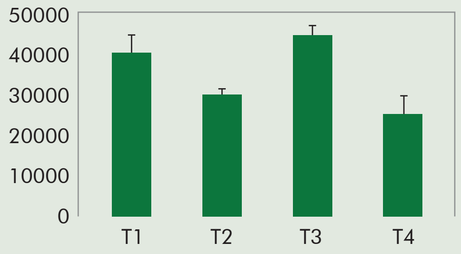


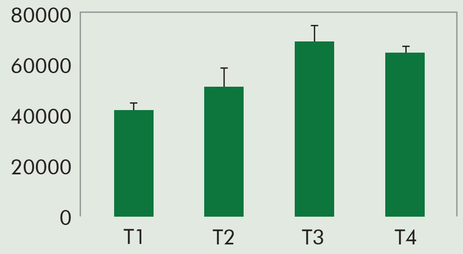


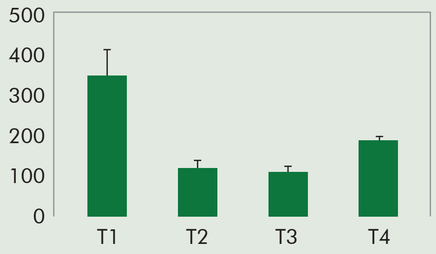




In order to provide further details on the effect of the Ca-based formulates (T2, T3, T4) on Ca distribution between external (apoplast) and internal (citosol) sites in plant cells, the OregonGreen fluorescent Ca-dye and Ca-biosensor (Cameleon YC3.6) probes were employed respectively on Arabidopsis plants, which represent a useful tools in plant biology to investigate molecular mechanisms of physiological processes.



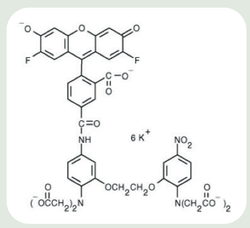


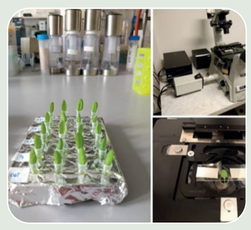


At Time 48 individual leaves were detached from the plants and directly analzyed with a wide-field microscope in case of the Cameleon expressing line or loaded with the low affinity and membrane-impermable Oregon Green™ 488 BAPTA-5N probe

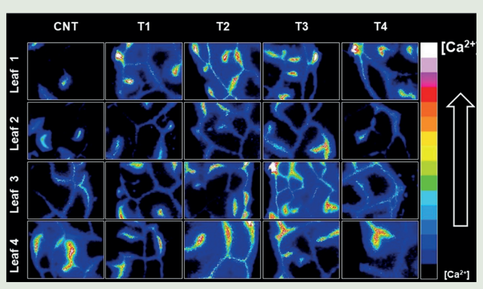
detection of apoplastic Ca^{2+}

the low affinity and membrane-impermeable Oregon Green™ 488 BAPTA-5N probe is a visible light excitable, low affinity Ca2+ indicator





Different phases of Oregon Green fluorescent Ca-dye staining in Arabidopsis leaves

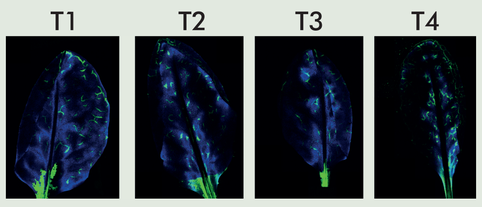


Wide-field based fluorescent of apoplastic and cytosolic calcium levels

Visualization of apoplastic Ca by using wide-field fluorescence microscopy imaging on portion of Arabidopsis leaves loaded with the Ca dye Oregon Green. CNT; negative control; T1, T2, T3 and T4 mean the four treatment used.

Confocal based fluorescent of apoplastic calcium levels

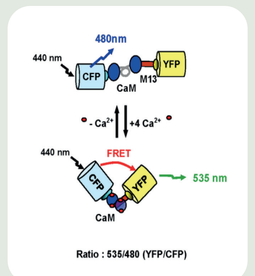
Visualization of apoplastic Ca by using confocal microscopy imaging on whole leaves Arabidopsis loaded with the Ca dye Oregon Green. T1, T2, T3 and T4 mean the four treatment. Oregon Green 5N (green signal); Chlorophyll (blue signal).



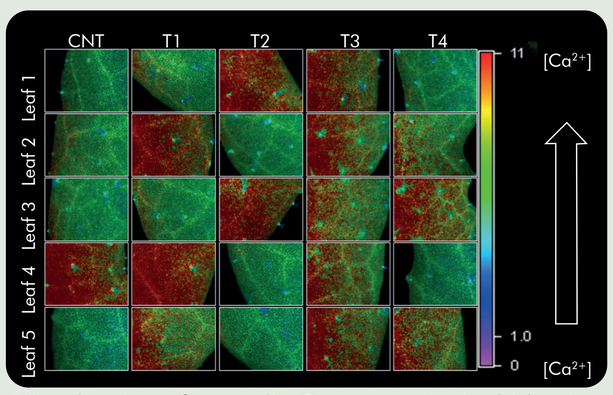
monitoring cytosolic Ca^{2+}

Ca^{2+} biosensors: CAMELEON

Cameleon biosensors are Föster Resonance Energy Transfer (FRET) based indicators in which the fluorescent protein cyan fluorescent protein (CFP) are linked to a yellow fluorescent protein (YFP or the circularly permutated variants, such as cpVenus) by the calcium-binding calmodulin protein. Binding of Ca^{2+} to calmodulin protein impact on the distance of CFP and YFP fluorophores and thereby on the FRET efficiency. Therefore, a single excitation and the simultaneous acquisition of two independent fluorescence emissions allows to determine a ratio proportional to the calcium concentration. In this work, Arabidopsis lines expressing CaMeleon-biosensor were used to monitor Ca dynamics at the mitochondrial (YC3.6 biosensor) and at cytosolic (NES) levels.

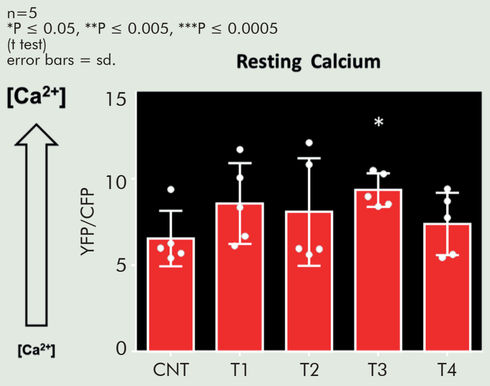


Effect of Ca-based formulates (T2, T3, T4) on Arabidopsis plant expressing Cameleon sensors (cytosolic Ca sensors, NES-YC3.6).



Visualization of cytosolic Ca content in Arabidopsis leaves by wide-field fluorescent microscopy

n=5
*P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0005
(t test)
error bars = sd.



Quantification of FRET signals of NES-YC3.6 biosensors in Arabidopsis leaves

CONCLUSIONS

LETTUCE

Application of GHI_789 on leaf displayed high variability resulting in a not significant variation in the content of Ca in lettuce when compared with all the treatments.

Application of GHI_049 on leaf leads to a Ca content increase in lettuce leaves (mainly in the apoplast fraction) when comparted with all the treatments considered

Application of $CaCl_2$ on leaf leads to a Ca content increase in all the fractions of lettuce leaves when comparted with all the treatments considered

ARABIDOPSIS

GHI_789 (T2) applications on Arabidopsis plants did not revealed differences in Ca distribution in the apoplast as well as in the cytosol when compared with T1 and CNT plants.

Application of GHI_049 (T3) on Arabidopsis leaves leads to an evident increase of Ca fluorescent signal in the apoplasm. Interestingly, GHI_049 formulates leads to an increase of Ca content in the cytosolic fraction compared with CNT plants.

Application of $CaCl_2$ (T4) on Arabidopsis leaves leads to an evident increase of Ca fluorescent signal in the apoplasm. However, $CaCl_2$ formulates did not affect the Ca content in the cytosolic fraction compared with CNT plants.