1.3.24 Plant molecular physiology

Molecular and post-genomic approaches to plant physiology provides indications of the mechanisms that drive the plant response to environmental stress and endogenous stimuli. In the NEST laboratory, NanoPLANT has carried out studies on plant molecular physiology, developmental biology, synthetic biology and contributed to nanostructure application to the agrochemical compartment.

NEST research center guarantees the possibility to conduct scientific projects in plant physiology with advanced methodologies of molecular and cellular biology. Measurements with laser scanning confocal microscopy join the possibility to perform quantitative assays for analysis of processes at nanoscale on living cells. These methods are complementary to genomic, transcriptomic, proteomic and metabolomic methodologies that characterize the expertise of NanoPLANT. In this way, we offer a large qualitative and quantitative overview of biological processes that are under study. This approach has contributed substantially to deepen basic knowledge in the field of plant developmental biology, molecular physiology and synthetic biology. In parallel, new nanostructures developed at NEST were tested on plant systems available at NanoPLANT in order to develop new applications for industrial use.

Plant survival is greatly impaired when O_2 levels are limiting, such as during flooding or when anatomical constraints limit O_2 diffusion. O_2 sensing in *Arabidopsis thaliana* is mediated by Ethylene Responsive Factor (ERF)-VII transcription factors, which control a core set of hypoxia- and anoxia-responsive genes responsible for metabolic acclimation to low-O_2 conditions. Anoxic conditions also induce genes related to reactive oxygen species (ROS). Whether the O_2-sensing machinery coordinates ROS production under anoxia has remained unclear. Recently, we showed that a low-O_2-responsive universal stress protein (USP), Hypoxia Responsive Universal Stress Protein 1 (HRU1), is induced by RAP2.12 (Related to Apetala 2.12), an ERF-VII protein, and modulates ROS production in Arabidopsis [1, Fig. 1].

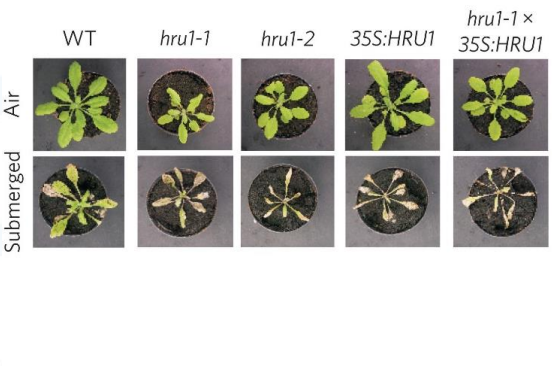

Figure 1. Effect of 4 days of submergence in the dark on the survival of Arabidopsis plants. The bar chart shows the percentage of surviving plants. Data and photographs were taken after 10 days of recovery in air. Letters indicate significantly different means (ANOVA, $p < 0.05$).

We found that HRU1 is strongly induced by submergence, but that this induction is abolished in plants lacking RAP2.12. Mutation of HRU1 through transfer DNA (T-DNA) insertion alters hydrogen peroxide production, and reduces tolerance to submergence and anoxia. Yeast two-hybrid and bimolecular fluorescence complementation (BiFC) analyses reveal that HRU1 interacts with proteins that induce ROS production, the GTPase ROP2 and the NADPH oxidase RbohD, pointing to the existence of a low-O_2-specific mechanism for the modulation of ROS levels.

We propose that HRU1 coordinates O_2 sensing with ROS signaling under anoxic conditions.
Our activities also focus on the characterization of molecular traits that promote plant tolerance to O_2 shortage and therefore may represent a major target for breeding purposes. In this context, following the identification of the calcineurin B-like interacting protein kinase 15 (CIPK15), which is a regulator of starch degradation in rice, the low O_2 signal elicited during rice germination under submergence has been linked to the sugar sensing cascade and calcium (Ca^{2+}) signaling. CIPK proteins are downstream effectors of calcineurin B-like proteins (CBLs), which act as Ca^{2+} sensors, whose role under low O_2 has yet to be established. We described CBL4 as a putative CIPK15 partner, transcriptionally activated under low O_2 in rice coleoptiles [2, Fig. 2]. The transactivation of the rice embryo CBL4 transcript and CBL4 promoter was influenced by the Ca^{2+} blocker ruthenium red (RR). The bimolecular fluorescence complementation (BiFC) assay associated to fluorescence recovery after photobleaching (FRAP) analysis confirmed that CBL4 interacts with CIPK15. The CBL4-CIPK15 complex is localized in the cytoplasm and the plasma-membrane. Experiments in protoplasts showed a dampening of α-amylase 3 (*RAMY3D*) expression after CBL4 silencing by artificial miRNA. Our results suggest that under low O_2, the Ca^{2+} sensor CBL4 interacts with CIPK15 to regulate RAMY3D expression in a Ca^{2+}-dependent manner.

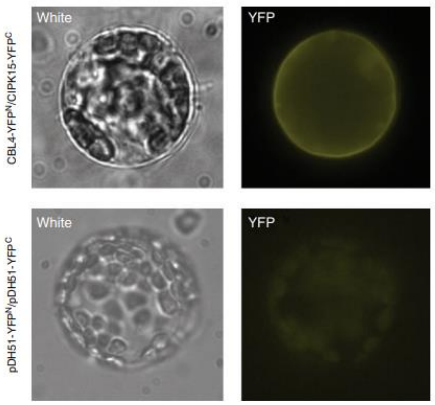

Figure 2. CBL4 interaction with CIPK15 in rice protoplasts. BiFC analysis of the protein–protein interaction between CBL4 with CIPK15. CBL4 was fused to the N-terminus of YFP (CBL4: YFPn), whereas CIPK15 was fused to the C-terminus of YFP (CIPK15:YFPc). The constructs were co-transformed into rice protoplasts. The pDH51-YFPn and pDH51-YFPc BiFC control plasmids were used as negative controls. The pictures are representative of four replicate experiments.

In mammals, the expression of hypoxia-response genes is controlled by the heterodimeric Hypoxia-Inducible Factor. The activity of this transcriptional regulator is linked mainly to the O_2-dependent hydroxylation of conserved proline residues in its α-subunit, carried out by prolyl-hydroxylases, and subsequent ubiquitination via the E3 ligase von Hippel-Lindau tumor suppressor, which targets Hypoxia-Inducible Factor-α to the proteasome. By exploiting bioengineered versions of this mammalian O_2 sensor, we designed and optimized a synthetic device that drives gene expression in an O_2-dependent fashion in plants, Fig. 3 [3].
Transient assays in Arabidopsis mesophyll protoplasts indicated that a combination of the yeast Gal4/upstream activating sequence system and the mammalian O_2 sensor machinery can be used effectively to engineer a modular, O_2-inducible transcriptional regulator. This synthetic device also was shown to be selectively controlled by O_2 in whole plants when its components were expressed

stably in Arabidopsis seedlings. We envision the exploitation of our genetically encoded controllers to generate plants able to switch gene expression selectively depending on O_2 availability, thereby providing a proof of concept for the potential of synthetic biology to assist agricultural practices in environments with variable O_2 provision.

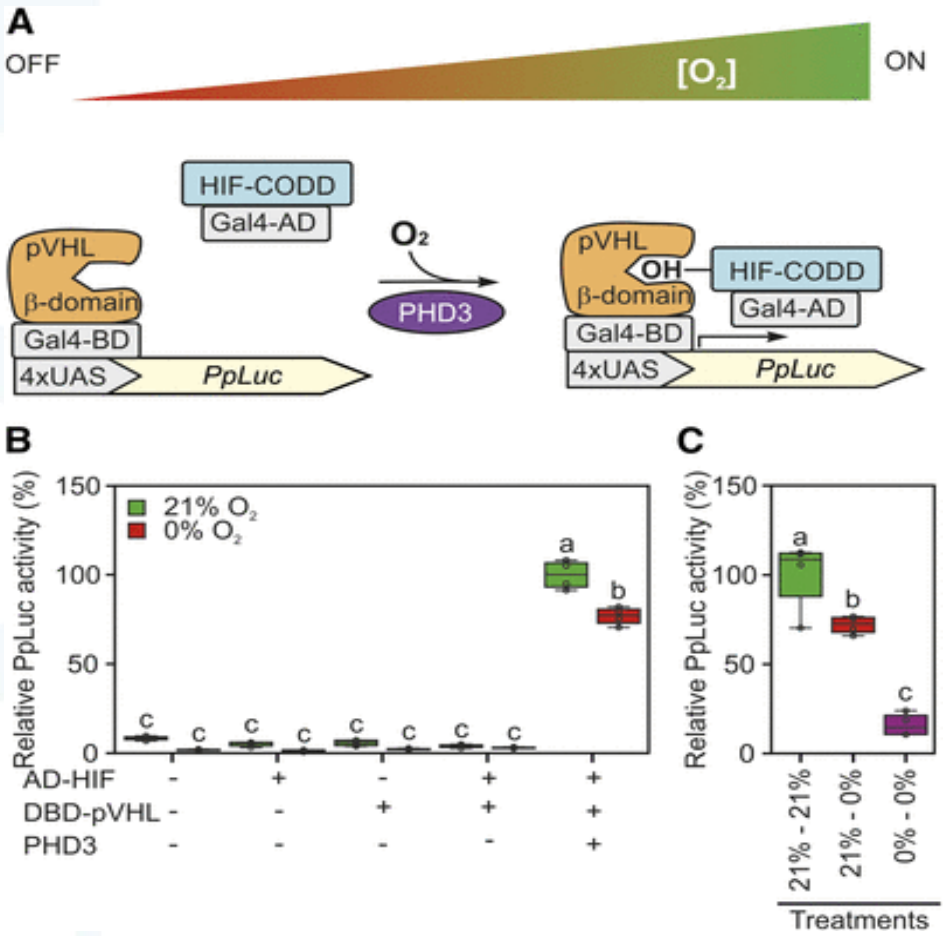

Figure 3. Schematization and testing of a synthetic O_2 sensor device based on the mammalian hypoxia sensing. A, Conceptual working mechanism of the device dependent on the environmental O_2 concentration. B, Sensor output in Arabidopsis protoplasts subjected to 18-h-long anoxia (0% O_2) or normoxia (21% [v/v] O_2) 12 h after transfection with plasmids bearing the modules depicted in A. C, Comparison of sensor responsiveness to an 18-h-long anoxic treatment applied either 12 h after protoplast transfection (21%–0%) or immediately after it (0%–0%).

While hypoxia is a detrimental and important component of flooding stress, we also found stable low O_2 conditions in plant meristems, the site of the plant stem cell pools. O_2 profiles in meristems were made using miniaturized O_2 electrodes or fluorescence-based microoptodes (Fig. 4a-b). Such conditions were confirmed at the NanoPLANT using laser scanning confocal microscopy by employing hypoxia biosensors. While low O_2 availability in stem cells may appear counterintuitive at first glance, such local hypoxic conditions were shown to play an important role in preventing differentiation and to regulate the rate at which new leaves are formed. A similar role for O_2 in directing pluripotency was also shown for several metazoan stem cells niches and different types of cancer, suggesting convergent evolution. Now we are further elucidating the molecular mechanism that acts to prevent differentiation in hypoxic environments and to

understand how low O_2 conditions are maintained at the meristems. To study this we are currently characterizing newly developed fluorescence based tools to measure O_2 levels at high spatial resolution in tissue (Fig. 4c-d). The most promising candidates of these rely on variable sensitivity to O_2 of specific fluorescent proteins. We also recently installed a microsensor multimeter setup, which aside from O_2, can also be used to measure H_2, H_2S, N_2O, NO, pH, Redox and Temperature.

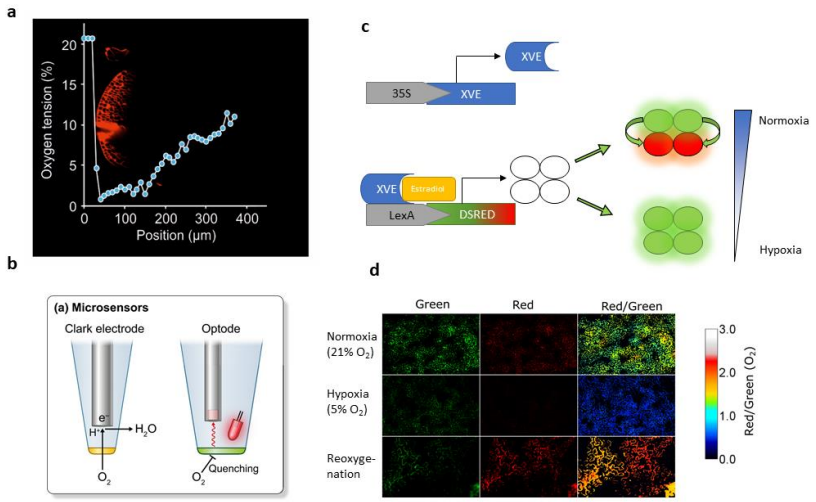

Figure 4. Role of O_2 in plant development. a) A representative O_2 profile through the shoot apical meristem showing that this stem cell pool is hypoxic. Confocal microscopy using FM4-64 membrane staining was used to detect the site of insertion of the O_2 microsensor. b) Representation of two types of O_2 microsensors: Clark type electrodes and phosphorescent based optodes. c) Schematic representation of the expression cassette of the DsRed FT based O_2 biosensor. A two-component inducible expression system employs a constitutively expressed XVE transcription factor, that upon binding to the steroid hormone estradiol activates expression of DsRed FT. The DsRed FT fluorophore can mature either to emit green or red fluorescence depending on the O_2 concentration. d) Green and red fluorescence of DsRed FT under different oxic conditions: normoxia, hypoxia and reoxyation. Normoxia permits green and red fluorescence, while hypoxia only shows green fluorescence.

References

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