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The next frontier: Exploring plant hypoxia sensing and response mechanisms through synthetic biology

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How can we comprehend the complexity of life at the cellular level when pathways and regulatory mechanisms are so inherently intertwined and redundant? This issue is at the core of every biological question and is both a hurdle and an opportunity in our endeavors to understand how cellular pathways function in a coordinated manner. One proposed approach to overcome this problem is to study pathways in so-called "orthogonal" systems through the use of "synthetic biology," a term coined in 1912 (1), long before the advent of molecular biology. This approach follows engineering principles for the reconstitution of a biological pathway in an organism or system that does not interact with that pathway (2). This is a complex task that requires prior knowledge, precise experimental design, and multidisciplinary research that includes the formulation of mathematical models to infer novel mechanisms. Lavilla-Puerta et al. (3) have reached a new high in the reconstitution of plant low oxygen (hypoxia) sensing and response mechanisms using the baker's yeast *Saccharomyces cerevisiae* to explore and identify new components and regulatory mechanisms that underpin hypoxia sensing and response in plants.

The field of hypoxia research in plants has made several leaps in the last two decades, from identifying essential oxygen sensing components to understanding core mechanisms underlying the genome-wide transcriptional reprogramming that allows the activation of hypoxia responses (4, 5). Hypoxia is now also established as being two-faced—i.e., physiologically

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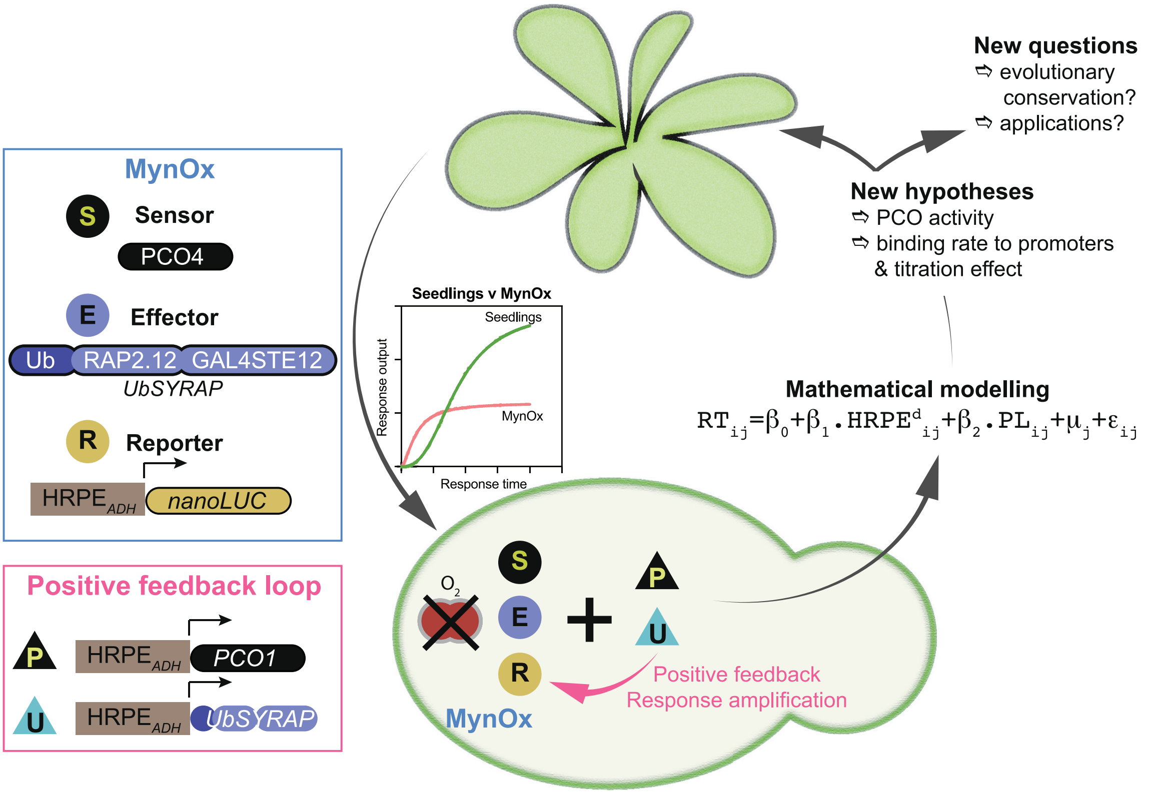

Fig. 1. The MynOx orthogonal system to study plant hypoxia sensing and response through a synthetic biology approach. Biochemical, molecular, and genetic information gathered by the plant hypoxia scientific community was used to design a sensor, an effector and a reporter that could be introduced in the yeast *S. cerevisiae*. Combined with the use of thin-layered yeast colonies, MynOx enabled the identification of positive feedback mechanisms in early hypoxia response. Mathematical modeling further inferred a particular relevance of PCO activity and ERFVII-mediated titration effects. This new information and hypotheses may be leveraged to better understand how plants sense and respond to hypoxia, with potential applications in terms of improving crop tolerance to flooding.

relevant but also a stress (termed "acute" hypoxia which typically occurs when plants experience flooding) (6, 7). While hypoxia responses have initially been studied largely in the context of flooding/acute hypoxia, recent findings have highlighted how physiological hypoxia is central to developmental processes, such as the maintenance of meristem function or the transition to flowering. These roles are achieved through the oxygen-dependent degradation of key regulators such as LITTLE ZIPPER 2 (ZPR2) and VERNALIZATION 2 (VRN2) via the ubiquitin-dependent N-degron pathway (8–10). Notably, this protein degradation pathway is also at the core of acute hypoxia sensing and the subsequent activation of the transcriptional response program.

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PLANT CYSTEINE OXIDASE (PCO) enzymes, whose activity is dependent on oxygen levels (11), act as direct oxygen sensors in plants and are a key component of the N-degron pathway through their oxidation of the N-terminal cysteine residue of substrate proteins (12). This oxidation can be followed by the substrate's N-terminal arginylation (i.e., conjugation of arginine at the N terminus of the substrate; catalyzed by Arg-transferases (13, 14)), then recognition by the E3 ubiquitin ligase PROTEOLYSIS 6 (PRT6) (15), and degradation by the proteasome. Notably, a similar mechanism exists in mammals via a cysteamine (2-aminoethanethiol) dioxygenase (ADO) enzyme (16). The above-mentioned VRN2 and ZPR2 are examples of physiological PCO substrates degraded through the N-degron pathway, whose stabilization in physiologically hypoxic tissues is essential for development. Another set of PCO and N-degron pathway substrates are transcription factors of the group VII ETHYLENE RESPONSE FACTOR family (collectively noted ERFVII), including RELATED TO APETALA 2.2 (RAP2.2), RAP2.3, RAP2.12, HYPOXIA RESPONSIVE ERF 1 (HRE1) and HRE2 (17, 18). These ERFVII transcription factors act as the master regulators of the acute hypoxia response program, placing them at the center of mechanisms involved in plant survival to flooding (19). Despite these advances many open questions remain such as the existence of regulatory loops or feedback mechanisms that might contribute to amplifying and stabilizing the early response to acute hypoxia, or the biological relevance of having both constitutively expressed and hypoxia-inducible PCOs and ERFVIIs (12, 20, 21).

Contrary to plants, the yeast *S. cerevisiae* senses oxygen via heme and ergosterol-dependent mechanisms (22). This evolutionary difference in oxygen-sensing mechanisms, combined with the absence of ERFVII transcription factors in yeast (23), and the conservation of the specificity of N-degron pathway components other than the PCOs (5), allowed Lavilla-Puerta et al. to establish a yeast orthogonal system—termed MynOx—that builds on previous synthetic biology approaches (24). As demonstrated by Lavilla-Puerta et al., MynOx can now be used as a tool to apply principles of synthetic biology with the aim of discovering and elucidating new mechanisms involved in oxygen sensing and in the regulation of plant responses to hypoxia.

MynOx: An Orthogonal System to Push the Boundaries of Knowledge

Lavilla-Puerta et al. first rationally designed and screened for optimal plant-based synthetic components that could be introduced into yeast to reconstitute plant hypoxia sensing and response in *S. cerevisiae* (Fig. 1). Specifically, MynOx includes an oxygen sensor (PCO4) and an effector (RAP2.12 fused to the GAL4STE12 domain and ubiquitin; called UbSYRAP). The latter translates the information from the oxygen sensor into a transcriptional output that is monitored using a nano-luciferase reporter (*HRPE*$_{ADH}$*:nanoLUC*), whose promoter contains hypoxia-responsive promoter elements (HRPEs) (19) that make it inducible by UbSYRAP under hypoxic conditions. Establishing MynOx also required identifying the best method to apply hypoxia to yeast cells, so that the oxygen levels and the dynamics of hypoxia response in MynOx would be similar to those in seedlings of the model plant *Arabidopsis thaliana*. An initial setback indicated that, in yeast, oxygen changes in the medium were too slow and did not follow the same pattern as in plants, but optimization steps showed that transferring yeast cultures to medium pre-equilibrated in hypoxic conditions or the use of thin-layered colonies (to avoid oxygen gradients) resulted in an output that was much closer to that found in *Arabidopsis* seedlings.

Combining the MynOx system with the thin-layered colony approach, the authors were then able to compare the output of the HRPE$_{ADH}$ reporter in Arabidopsis seedlings and in MynOx. This revealed differences in the timing of HRPE$_{ADH}$ induction, with yeast providing a faster reporter response than seedlings. Notwithstanding this difference, a more striking observation was that the amplitude of the output was higher in Arabidopsis seedlings than in yeast, in which the steady-state was reached faster, but also remained at a lower level than in seedlings. These differences suggested that additional factors in plants play an important role in hypoxia response amplification and maintenance. Thus, MynOx could be used to infer the existence of a potential positive feedback loop downstream of hypoxia sensing.

Addition of a Positive Feedback Loop Improves the Output of MynOx

Building on previous knowledge of constitutively expressed or of hypoxia-inducible sensors and effectors, Lavilla-Puerta *et al*. hypothesized that the presence of hypoxia-inducible PCOs (e.g., *PCO1*) and ERFVIIs (e.g., *HRE1* or *HRE2*) could constitute a positive feedback mechanism for signal amplification in plants. MynOx enabled them to test this possibility without interference with other potential in planta feedback mechanisms (Fig. 1). To add the putative positive feedback mechanism to yeast, *PCO1* and another copy of *UbSYRAP* were introduced under the control of the UbSYRAP-inducible *HRPE*$_{ADH}$ promoter. Notably, addition of both *HRPE*$_{ADH}$*:PCO1* and *HRPE*$_{ADH}$*:UbSYRAP* to MynOx allowed enhancement of the dynamic range of MynOx response to hypoxia. Another important finding was that, when added individually, introduction of the hypoxia inducible $HRPE_{ADH}$:*UbSYRAP* played a more important role in response amplification than $HRPE_{ADH}$:*PCO1*.

Mathematical Modeling and Predictions

Next, using previously obtained biochemical data on the different components of MynOx, Lavilla-Puerta et al. built four different mathematical models to generate predictions, lighting the path to the next questions (Fig. 1). Notably, their models predict that i) the existence of a hypoxia-inducible module for the expression of ERFVII transcription factors contributes to overcoming remaining PCO activity under hypoxic conditions; ii) the binding rate of UbSYRAP (and hence possibly of ERFVIIs in planta) to HRPEs has a strong effect on the transcriptional outputs; iii) in the presence of the positive feedback loop, a competition between HRPE-containing promoters may exist, thus enabling a range of response outputs, as presumed based on transcription factor titration effects (25); iv) the regulation of PCO1 activity appears to be a key component in the system, such that higher PCO1 activity under hypoxia may contribute to a faster response.

Where to Now? The Next Frontiers…

The development of the orthogonal MynOx system and its use has led to new knowledge and hypotheses that can now be tested both in the orthogonal system and in planta, thus allowing the field of plant hypoxia research to reach the next frontier. Another new frontier lies in the further development of the MynOx system by, for example, adding other known components of the plant hypoxia sensing and response pathway to analyze their impact one by one or in combination. For example, MynOx could be used to explore the roles of negative regulators of ERFVIIs, which are generally less understood (26). Notably, the use of the MynOx system may not be limited to understanding how plants sense and respond to hypoxia. Perhaps MynOx could be "tweaked" to also study hypoxia response in animal systems, especially those dependent on the activity of ADO.

The findings here also bring new questions in terms of the evolutionary conservation of the proposed new mechanisms in plants (27) and their application to improving crop tolerance to flooding via the manipulation of oxygen-dependent enzymes such as PCO1. Finally, this study has broader implications than our understanding of hypoxia response in plants, and highlights more generally the value of combining synthetic biology, mathematical modelling, and "classical" biology *to explore strange new [mechanisms], to seek out new life [science frontiers], to boldly go where no one has gone before!*

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