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Running title: A synthetic oxygen sensing device for plants

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Title: A synthetic oxygen sensor for plants based on animal hypoxia signalling

One sentence summary: ***A biological device consisting of mammalian, fungal, and plant components drives gene expression in an oxygen-dependent manner.***

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Author contributions

S.I., B.G. and F.L. conceived the project and planned the research activities, with critical suggestions by P.P.; S.J. and L.C. carried out bioinformatics analyses of protein structures under the supervision of B.M.; S.I. performed the experiments with the technical assistance of L.P., B.G., F.C. and F.L.; P.P. ensured funding for the experimental activities; S.I., B.G. and F.L. wrote the article.

Abstract

Due to the involvement of oxygen in many essential metabolic reactions, all living organisms have developed molecular systems that allow adaptive physiological and metabolic transitions depending on oxygen availability. In mammals, the expression of hypoxia-response genes is controlled by the heterodimeric Hypoxia-Inducible Factor (HIF). The activity of this transcriptional regulator is mainly linked to the oxygen-dependent hydroxylation of conserved proline residues in its α subunit, carried out by Prolyl-Hydroxylases (PHDs), and subsequent ubiquitination via the E3 ligase von Hippel–Lindau tumor suppressor (pVHL), which targets HIF-α to the proteasome. By exploiting bioengineered versions of this mammalian oxygen sensor, we designed and optimized a synthetic device that drives gene expression in an oxygen-dependent fashion in plants. Transient assays in *Arabidopsis thaliana* mesophyll protoplasts indicated that a combination of the yeast Gal4/UAS system and the mammalian oxygen sensor machinery can be effectively used to engineer a modular, oxygen-inducible transcriptional regulator. This synthetic device was also shown to be selectively controlled by oxygen in whole plants, when its components were stably expressed in Arabidopsis seedlings. We envision the exploitation of our genetically encoded controllers to generate plants able to switch gene expression selectively depending on oxygen availability, thereby providing a proof-of-concept for the potential of synthetic biology to assist agricultural practices in environments with variable oxygen provision.

key-words: hypoxia, prolyl hydroxylases, Gal4, plant synthetic biology, genetically encoded reporters

Introduction

As sessile organisms, plants must adapt their developmental and metabolic programs to the surrounding environment, especially when this entails stress conditions. Such adaptation is often achieved through the perception of exogenous and endogenous stimuli via dedicated sensors, which trigger signalling cascades that, in turn, culminate in the reprogramming of gene expression and metabolism. Numerous proteins involved in the sensing of temperature (Jung et al., 2016), light quality and quantity (Possart et al., 2014), water availability (Zhu, 2002) and nutrient deficiency (Cui, 2012) have been identified in *Arabidopsis thaliana* and other plant species, and are currently being investigated to unveil their signalling mechanisms.

Plants successfully colonized most terrestrial niches by evolving specific adaptive traits to stress conditions, depending on the duration, intensity and frequency at which these adverse conditions occur in different regions of our planet. However, the relatively recent transfer of crops from their area of origin and extreme climatic events cause serious limitations to productivity, since cultivated plants are exposed to environmental conditions for which they have not yet evolved any adaptations (Crane et al., 2011; Pingali, 2012; Nelson et al., 2014). To this end, breeding using simple beneficial traits may not be sufficient to rapidly develop stress-resistant crop varieties. The engineering of complex and novel traits using strategies inspired by synthetic biology, based on the fast advances in plant molecular physiology, represents a promising way to overcome such limitations. Synthetic biology strategies aim at reconstructing artificial genetic circuits, often of modular structure and composed of functionally characterized constituents, that are able to operate without interfering with the host cell functions ("orthogonally") (Baltes and Voytas, 2015; Liu and Stewart, 2015). Successful design of orthogonal drought responses (Yang et al., 2010), synthetic nitrogen fixation (Florence, 2016) or improved photosynthetic performances (Erb and Zarzycki, 2016) have shown the remarkable potential held by such approaches.

Among the adverse environmental conditions that limit yield production, flooding is one of the main causes for agricultural losses worldwide (Bailey-Serres et al., 2012). Since gas diffusion is extremely reduced in water as compared with air (Armstrong, 1980), submerged plant tissues are exposed to the concomitant decrease in oxygen availability and accumulation of CO_2 and ethylene, which inhibit aerobic metabolism (Bailey-Serres and Voesenek, 2008). Considering also the reduced efficiency of photosynthesis in muddy water (Mommer and Visser, 2005), flooded or waterlogged plants suffer of a general energy crisis that, if prolonged, can lead to death (Licausi and Perata, 2009). To date, crop improvement approaches aimed at enhancing resistance to transient flooding have been mostly applied to rice (*Oryza sativa*), where either fast elongation determinants

during germination (Kretzschmar et al., 2015) or arrest of underwater growth in adult plants (Xu et al., 2006) have been exploited. Remarkably, this last strategy relies on transcriptional regulators dedicated to the metabolic adaptation to hypoxia in plants: the Group VII Ethylene Response Factor (ERF-VII) proteins (Giuntoli et al., 2018).

Surprisingly, the same class of transcription factors has been shown to participate in the response to other environmental stimuli, including nitric oxide (Gibbs et al., 2014), sugar status (Loreti et al., 2017), and developmental processes (Abbas et al., 2015; Meitha et al., 2018). Therefore, efficient engineering of flood-adaptive strategies, avoiding an effect on the overall plant physiology, requires the development of a strategy to relate specific gene expression to oxygen availability in an orthogonal manner.

In order to evaluate the feasibility of this strategy, we explored possible alternative strategies of oxygen sensing developed by other organisms. In most animal cells, transcriptional adaptation to low oxygen conditions is mediated by Hypoxia-Inducible Factor (HIF) basic helix-loop-helix (bHLH) regulators. This oxygen sensing mechanism is based on hydroxylation of specific Pro residues carried by HIF1α (Pro402 and Pro564) (Ivan et al., 2001), catalysed by oxygen and oxoglutarate-dependent enzymes (PHDs, Prolyl-hydroxylases) (Epstein et al., 2001, Bruick and McKnight, 2001). The hydroxylated HIFα is subsequently polyubiquitinated by a ubiquitin E3 ligase complex, which contains the substrate recognition subunit pVHL (Maxwell et al., 1999). Polyubiquitination leads to HIFα degradation through the 26S proteasome complex. When oxygen levels drop, HIFα is protected from proteolysis and thereby enabled to migrate into the nucleus (Depping et al., 2008). Here, heterodimerization with its cognate β-subunit (Wang et al., 1995) reconstitutes a functional transcription complex able to induce the expression of hypoxic genes.

In humans, a single HIFβ isoform and three different HIFα isoforms have been identified (Ema et al., 1997; Gu et al., 1998). Among them, HIF1α and HIF2α have been extensively investigated and are currently considered to act as the master regulators of the hypoxic response. In the case of PHD enzymes, three different isoforms have been identified (Bruick and McKnight, 2001). They differ for tissue specificity (Hirsilä et al., 2003), cellular localization (Metzen, 2003) and affinity for HIFα (Masson et al., 2001).

Numerous *in vitro* and *in vivo* assays have revealed the bases for the oxygen-dependent HIF/pVHL interaction. HIF1α contains two O_2-dependent degradation domains (ODD), called NODD or CODD depending on their proximity to the N-terminal and C-terminal end of the protein, respectively (Masson et al., 2001). The ODDs allow interaction with both pVHL (Ivan et al., 2001; Jaakkola et al., 2001) and PHDs (Bruick and McKnight, 2001; Epstein et al., 2001). In turn, pVHL activity relies on two different domains: an α-domain (spanning through residues 157-171) that

115 recruits the ubiquitination complex, and a β-domain (residues 63-157) involved in the HIF-ODD
116 interaction (Stebbins et al., 1999; Hon et al., 2002; Min et al., 2002).
117 Previous studies have shown that the HIF-pVHL interaction can be promoted in a heterologous
118 yeast system, provided that PHD is present (Alcaide-German et al., 2008). In the present work, we
119 developed a transcriptional regulatory device, based on the HIF-pVHL-PHD triad, able to switch
120 from an active to inactive state in response to oxygen availability in plants.
121
122

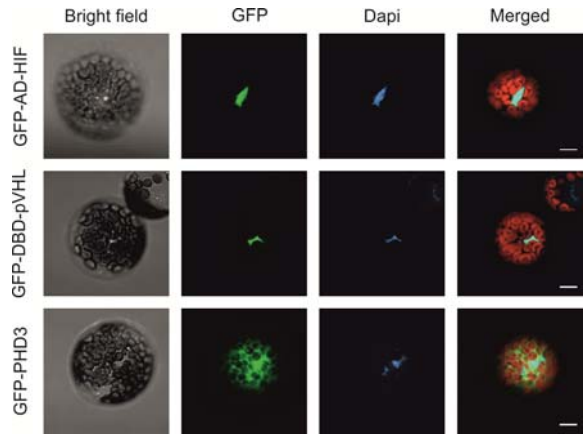
Results

Engineering an oxygen-sensing HIF-pVHL-PHD device in Arabidopsis protoplasts

To achieve orthogonal control of gene expression in plants in an oxygen-dependent manner, we took advantage of the mammalian HIF1α and pVHL pair. We selected the essential domains required for their oxygen-driven interaction, namely a CODD and a β-domain fragment encoded by $HIF1\alpha_{554-576}$ and $pVHL_{63-157}$. To reconstitute a fully functional transcriptional activator, we fused the HIF1α and pVHL sequences to the activation domain (AD) and the DNA binding domain (DBD) of the *Saccharomyces cerevisiae* Gal4 transcription factor (Keegan et al., 1986) to exploit a mechanism of intermolecular complementation of Gal4 function. In this way, we generated two chimeric effector modules: AD-HIF and DBD-pVHL. Since their interaction is expected to rely on HIF1α hydroxylation at the Pro_{564} residue (HIF_{Hyp}), the oxygen sensing device had to be equipped with a further unit, constituted by the human Prolyl-Hydroxylase 3 (PHD3) enzyme (sensory module). Our choice fell on this PHD isoform due to its high activity on the CODD substrate (Appelhoffl et al., 2004) and its relatively small size, which confers a homogenous distribution among cellular compartments, including the nucleus (Fig. 1). This ensured co-localization of PHD3 with AD-HIF and DBD-pVHL, both provided with a nuclear localization sequence (Fig. 1).

We therefore envisioned that the presence of oxygen would lead to AD-HIF hydroxylation, association of the effector modules and formation of a transcriptional complex. This, in turn, would be able to induce promoters containing the Gal4-specific *cis*-element, called the upstream activating sequence (UAS) (Guarente et al., 1982). Accordingly, we generated an output module by cloning the firefly (*Photinus pyralis*) luciferase reporter gene downstream of a synthetic UAS promoter (Fig. 2A). We also placed a second luciferase gene from sea pansy (*Renilla reniformis*) under the control of a constitutive *CaMV* 35S promoter, to be used as a normalization variable proportional to the transformation efficiency. Consequently, relative luciferase activity was measured as the readout of active complex formation, arising from the HIF_{Hyp}-pVHL interaction.

We tested the four modules in plant cells. The constructs were transiently expressed, in different combinations, in freshly prepared protoplasts from Arabidopsis mesophyll cells. Twelve hours after the transformation, protoplasts were either subjected to anoxic treatment or maintained in normoxia. The two effector proteins, when expressed alone, did not manifest trans-activation capacity, both in ambient and anoxic conditions (Fig. 2B). More strikingly, no output was produced by combination of the effectors without concurrent PHD3 expression, revealing that plants do not possess any endogenous enzyme able to hydroxylate the Pro_{564} residue borne by the HIF-CODD (Fig. 2B).

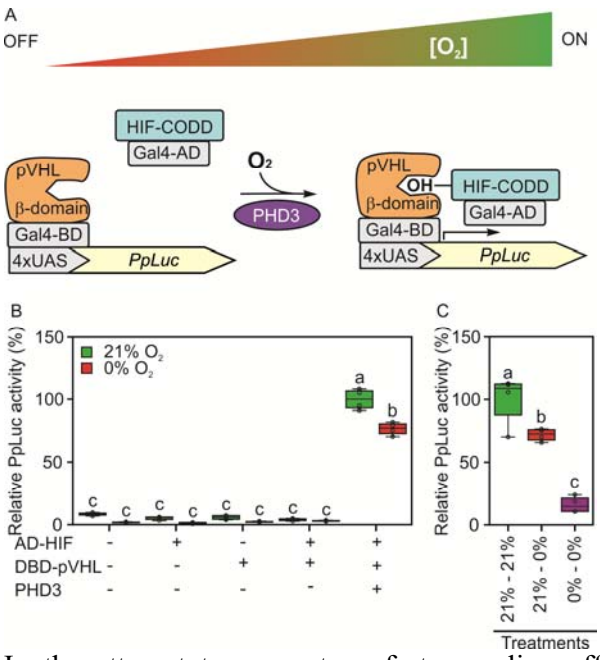


Instead, we detected increased relative luminescence in aerobic protoplasts expressing the full set of sensor modules (Fig. 2B), indicating that the chosen PHD3 isoform enabled AD-HIF interaction with DBD-pVHL and subsequent promoter activation.

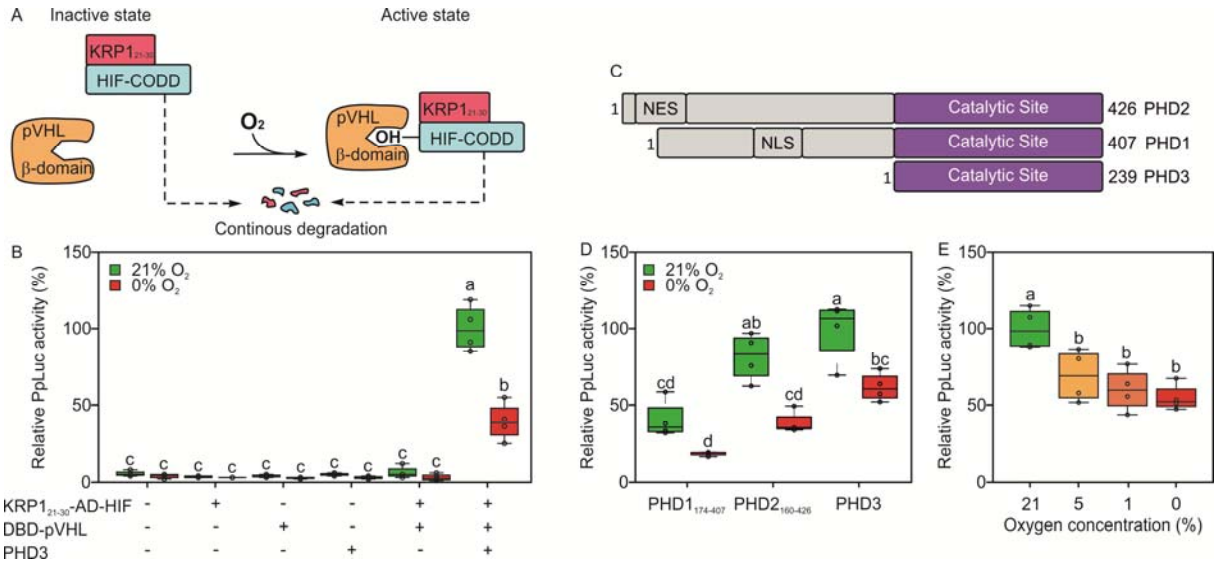
However, contrary to the expectations, prolonged exposure to complete anoxia exerted only a mild although significant repression of the luciferase reporter (Fig. 2B). Imposition of an anoxic atmosphere in the absence of photosynthetic activity (as a consequence of darkness) was assumed to deplete the intracellular oxygen availability below PHD affinity (Ehrismann et al., 2007). Excluding the residual catalytic activity of PHD3 under such conditions, we put forward that a slow turnover of the two interacting partners would result in the persistence of a fraction of the complex, sufficient to drive transactivation in the absence of oxygen. To evaluate this possibility, we subjected protoplasts to an immediate oxygen deprivation after transfection. In this case, we hypothesized that direct exposure of any newly formed HIF-CODD protein to an anoxic environment should prevent complex formation, thereby hindering promoter induction. Immediate exposure to the anoxic atmosphere limited the response of the output sensor considerably, when compared to the level displayed after sequential aerobic and anoxic incubations (Fig. 2C). Thus, we concluded that, once formed under oxygen-replete conditions, the HIF-pVHL effector complex is characterized by a relatively long half-life, resulting in a substantial amount of transactivating complex being still present and functional during the following anaerobic treatment.

Expanding the dynamic range of the oxygen sensor

In order to increase the hypoxic responsiveness of our oxygen sensor, we decided to enhance the turnover of at least one of the three components. In principle, AD-HIF could work as the main determinant of the activation state of the device, since its hydroxylation determines whether the transcriptional complex assembles. It is then conceivable that, during the progression of the anoxic treatment, a fast recycling AD-HIF would only leave newly synthesized units, which would no longer be functional.



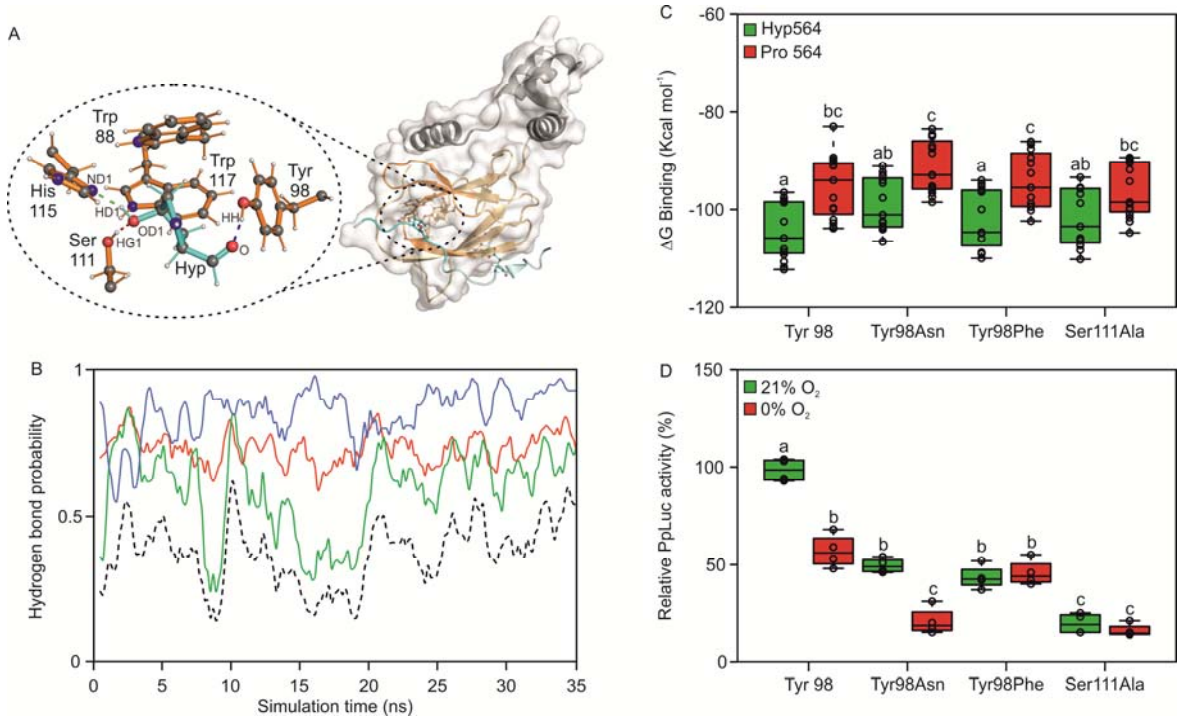
183 In the attempt to generate a fast recycling effector, we fused the N-terminus of the AD-HIF
184 chimeric protein to a known destabilizing domain, consisting of amino acid residues from 21 to 30
185 of the KIP-RELATED PROTEIN 1 (KRP1) (Li et al., 2016) of Arabidopsis (Fig. 3A). This new
186 version of the effector was tested in Arabidopsis mesophyll protoplasts. While retaining the ability
187 to be specifically activated by oxygen in the presence of PHD3, this time the sensor indeed showed
188 a larger reduction of promoter activity (-60% of the aerobic output) in anoxia-treated protoplasts
189 (Fig. 3B), as compared with the first module evaluated (Fig. 2B, -25% of the aerobic output). As
190 shown by direct comparison of the two HIF modules (Supplemental Fig. S1), the output was lower
191 in the presence of the modified HIF than when the original effector was used. Therefore, we
192 concluded that conjugation of the KRP1 domain modified the steady-state level of the effector
193 complex, decreasing its stability both in aerobic and in anoxic conditions. Nonetheless, more
194 pronounced destabilization in anaerobiosis determined an actual expansion of the dynamic range of
195 the sensor. Overall, this effect could be explained by the continuous degradation of KRP1-AD-HIF,
196 resulting in limited persistence of the active complex, which could only accumulate under aerobic
197 conditions when newly synthesized AD-HIF is hydroxylated by PHD3.
198
199 **Performance of the device under different magnitudes of the oxygen stimulus and with**
200 **alternative sensory modules**
201 Once we improved the mammalian-based oxygen device, we decided to evaluate its performance
202 when the PHD3 sensory module was replaced by the other prolyl hydroxylase isoforms annotated in
203 the human genome. The catalytic domains of PHD1 and PHD2 (Fig. 3C and Supplemental
204 Information S1) were tested in the protoplast system. When $PHD1_{174\text{-}407}$ was employed, the device



produced a reduced output as compared with the one associated to PHD3, due to lower aerobic reporter activity and decreased range (Fig. 3D). $PHD2_{160-426}$ had, instead, comparable performance to PHD3 (Fig. 2D), suggesting it as a suitable potential substitute of the latter in this kind of device. Additionally, we explored the sensor sensitivity to distinct atmospheric oxygen levels, as we envision that the ability of the HIF-pVHL sensor to discriminate between normoxia and hypoxia would be a necessary condition for its application as a proxy for physiological hypoxia in plants. To test this aspect, we incubated protoplasts expressing the complete sensor under a range of oxygen concentrations. Significant reduction of luciferase activity was recorded when comparing aerobic protoplasts to those subjected to oxygen limitation, but no significant difference was measured among samples treated with atmospheres containing either 5% or 1% O_2 (V/V), or totally devoid of it (Fig. 3E). We concluded that the synthetic sensor had high sensitivity to relatively mild decreases in O_2 availability (represented by 5% O_2 in our test), without being able to sense the magnitude of hypoxia further. Based on the responsiveness of the HIF-pVHL sensor to moderate drops in intracellular hypoxia, we propose it as a viable candidate to report about the occurrence of physiological hypoxia in living plant tissues.

In silico prediction and testing of mutated variants

Despite the employment of a fast recycling HIF module, basal activity of the sensor was still observed at the end of the anaerobic incubation (Fig. 3B), indicating that a substantial amount of the trans-activating complex was maintained after removal of oxygen. Therefore, we sought to weaken the HIF-pVHL interaction to further reduce the half-life of the active complex. To do so, we adopted a rational mutagenesis approach based on available structural information. Inspection of the three-dimensional structure of a pVHL/HIF-CODD complex, provided by Min et al. (2002) and



Hon et al. (2002), revealed that the association between the HIF fragment and pVHL not only involves interactions generated as a consequence of Pro564 hydroxylation (Fig. 4A), but also occurs through other residues. In the aerobic HIF_{Hyp}-pVHL complex, HIF1α Hyp564 lies in a deep pocket, lined up by pVHL residues Trp88, Tyr98, Ser111, His115 and Trp117, and establishes a hydrogen bond network with Ser111, His115 and Tyr98 (Fig. 4A). Additionally, HIF-CODD Asp571 and pVHL Arg107 form a salt bridge outside of the pocket accommodating Hyp564 (Supplemental Fig. S2A).

Impairment of these key interactions is expected to facilitate the dissociation of the complex. We speculated that a decreased complex stability, while being compensated by continuous assembly of new transcriptional units under aerobic conditions, might especially constrain output production under anoxic conditions.

We applied a molecular dynamic simulation to model distances and probability of occurrence of the pairwise interactions between HIF1α Hyp564 and the three aforementioned residues belonging to the pVHL pocket. The computational results revealed fluctuations of the hydrogen bond pattern over the simulation time and identified the hydrogen bond between the HIF_{564} β-carbon and pVHL Tyr98 as the most stable interaction (Fig. 4B).

Once we described the network of non-covalent interactions centred on HIF_{564}, we proceeded to the rational mutagenesis of the module. As a first target site, we focussed on pVHL Tyr98: the absence of other interactions, beyond the one involving HIF_{564}, together with its side chain orientation pointing outward from the surface of the complex made its substitution unlikely to interfere with

protein folding. An alternative target residue selected was Ser111; as its side chain was oriented inwards, it was exchanged with a short and neutral side chain (Ser111Ala) to abolish the interaction. The Tyr98Asn, Tyr98Phe and Ser111Ala variants were selected for molecular mechanics combined with generalized Born and surface area continuum solvation analysis (MM-GBSA), to predict changes in their relative binding energy with HIF_{564}. Tyr98Asn, a common mutation in many types of tumours (Knauth et al., 2006), had been shown before to destabilize the interaction. Substitution with Phe should provide the same structural properties as the wild type Tyr residue, only hindering hydrogen bond formation (Supplemental Fig. S2B). In general, the MM-GBSA did not predict a reduction in complex stability for the selected variants, suggesting the potentiality for complex formation for any of the selected variants (Fig. 4C). Tyr98Asn and Ser111Ala were predicted to be the most destabilizing variants since no statistical differences were found comparing their ΔG of binding measured in presence of HIF_{Hyp} with that of the WT variant in the presence of HIF_{Pro}, representing a complete dissociated complex (Fig. 4C).

We then tested experimentally these variants in the transient protoplast system. According to the output of the sensor, all point mutations on pVHL led to weaker interaction with HIF_{Hyp}, with Ser111Ala being the most destabilizing substitution (Fig. 3D). Tyr98Asn and Tyr98Phe variants were associated to a 50% loss of the aerobic output of the wild type sensor. Remarkably, only the Tyr98Asn preserved the oxygen regulation of the device and even showed an increased dynamic range compared to the wild type (Fig. 4D).

Due to this partial absence of correlation between MM-GBSA and experimental data, we decided to carry on with the *in vivo* investigation of the relevance of selected residues to the HIF-pVHL interaction. We thus evaluated the performance of an additional set of residues whose side-chains possess mildly or dramatically different physico-chemical properties. Specifically, three mutations (Tyr98His, Tyr98Trp, Tyr98Cys) were predicted to disrupt the hydrogen bond with HIF1α Hyp564, whereas another three (Tyr98Asp, Tyr98Glu and Tyr98Ile) had the potential to retain it (Supplemental Fig. S2B). We also examined the contribution of the remaining contact point between the two protein fragments as revealed by the crystal structure, the one formed by pVHL Arg107 and HIF-CODD Asp571. All new variants resulted in a general reduction of the aerobic interaction between the effector modules (Supplemental Fig. S2C). This was unexpected for the HIF_{571}-$pVHL_{107}$ interaction; although far apart from the pocket allocating Hyp564, it seemed instead to be crucial for active complex stability and conformation maintenance of the pocket. Overall, the trans-activation values recorded for $pVHL_{98}$ variants under aerobic conditions did not appear to correlate with the predicted ability of the substituted residues to form a hydrogen bond with the β-carbon of HIF_{564} (Fig. 4D and Supplemental Fig. S2B). The Tyr98Cys variant conserved

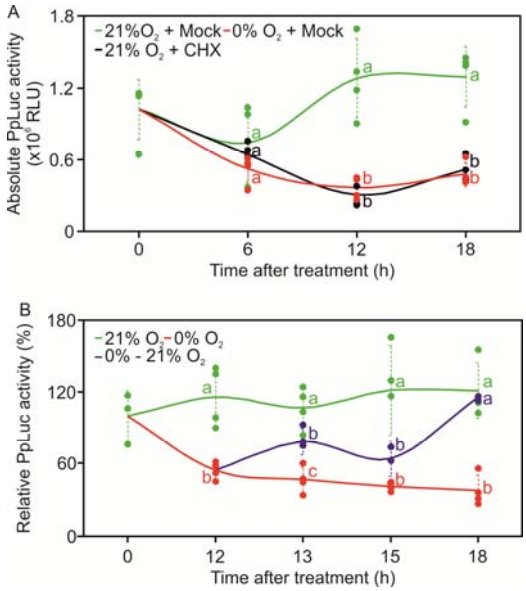
a moderate interaction capacity under aerobic conditions, although, as observed for Tyr98Phe, this interaction became independent from oxygen availability (Supplemental Fig. S2C). Strikingly, the oxygen regulation of the sensor was lost as a consequence of each individual point mutation, with the exception of Tyr98Glu, where it was nonetheless associated to lower stability of the complexes (Supplemental Fig. S2C).

Taking these results together, we could conclude that the responsiveness to oxygen of our device was ensured, beyond HIF-CODD Pro564, by several crucial residues, likely granting the maintenance of a proper conformation of the two effectors. It can be speculated that PHD3 access to the HIF_{564}-containing pocket was determined both by steric features of the pocket itself (relying on $pVHL_{98}$, $pVHL_{111}$ and HIF_{564}) and by faraway residues (such as $pVHL_{107}$ and HIF_{571}). Regarding the performance of the synthetic sensor, the most optimized version, among those examined in the transient test system (Arabidopsis mesophyll protoplasts), turned out to be composed by a constitutively destabilized form of the HIF-CODD peptide (KRP1-AD-HIF) in combination with the wild type form of the pVHL β-domain (DBD-pVHL).

Sensor output dynamics under reversible oxygen inputs

The persistence of luciferase activity observed under anaerobic conditions led us to wonder whether the high stability of the reporter protein could be masking a rapid inactivation of the device after removal of the stimulus (oxygen). Therefore, we decided to investigate the behaviour of the sensor in aerobic protoplasts in which *de novo* reporter synthesis was blocked and compare its output with the reduction of reporter activity observed in anaerobic protoplasts (Fig. 3B). We transformed the test system with all four modules constituting the sensor. This time, to follow the dynamic behaviour of the device and to assess the time of anoxia exposure required to turn it into an inactive state, we subjected protoplasts to anoxia for 6, 12 and 18 hours, after a 12 h aerobic incubation to ensure expression of all components. In parallel, we treated a subset of aerobic protoplasts with 100 μM cycloheximide (CHX), to block any *de novo* synthesis of the effector and reporter proteins and thereby gather information about the luciferase reporter half-life.

The sensor output remained substantially unaltered across the three aerobic time points, suggesting that the reporter protein was kept at a steady-state level during the investigated time frame (Fig. 5A). No significant difference in the output values was observed after 6 h among protoplasts treated with either anoxia, normoxia, or CHX, indicating the impossibility to determine a reduction in reporter activity in such range (Fig. 5A). In particular, the pattern displayed by CHX-treated cells revealed that full turnover of the reporter protein only took place between 12 and 18 hours after the block of protein synthesis. The turnover rate measured entails that, as long as a wild-type firefly



luciferase protein is used as the output, our transcriptional sensor could display responses to anoxia (i.e. output repression) with a minimum lag of 12 hours. Remarkably, a perfectly overlapping trend was observed for CHX-treated and anoxic protoplasts. Anoxia, comparably to CHX application, is expected to prevent novel HIF-pVHL active complex formation and indeed it caused a significant decrease in luminescence only after 12 h, with no further decrease at the last time point (Fig. 5A). These pieces of evidence clearly demonstrated that our oxygen-dependent device had reached a completely inactive state within 12 h from the onset of oxygen deprivation. In other words, the data suggest that the HIF-pVHL complex assembled during prior aerobic incubation had been processed within such time frame, while the newly synthesized modules had not been able to associate together.

After we explored the anoxia-dependent inhibition of the sensor, we moved on to evaluate its reversibility: for this purpose, anoxia-treated protoplasts were transferred back to aerobic conditions for 1, 3 or 6 h. Re-oxygenation started after 12 h of anoxia, as the end-point of sensor inactivation. Full restoration of control levels occurred after 6 h in air, but a response by the sensor was already detected as early as 1 h after re-oxygenation (Fig. 5B). Such a fast induction dynamic was in favour of the interpretation of a quick re-assembly of the pre-existing components (HIF_{Pro} and pVHL) into an active complex. In conclusion, our data show that the HIF and pVHL effectors produced in the absence of oxygen cannot interact, but a HIF-pVHL complex is readily re-formed as soon as the input is restored.

The HIF/pVHL transcriptional complex does not affect plant endogenous transcriptional regulation

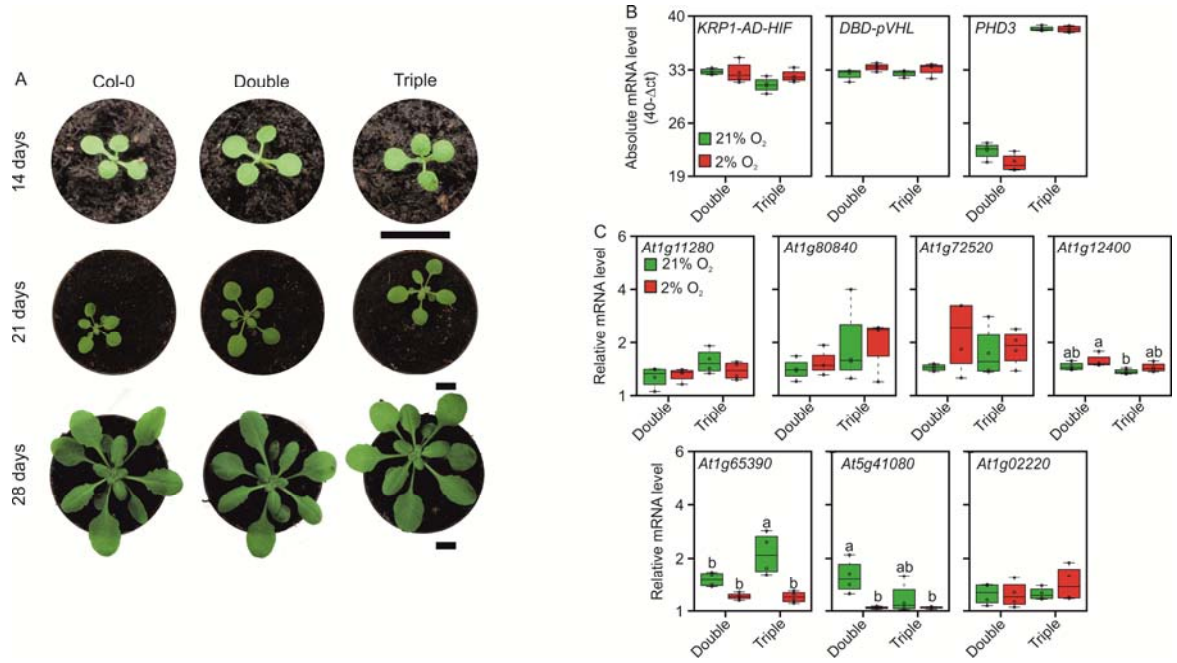
After describing it in a transient test system, we attempted to incorporate the genetically encoded synthetic oxygen sensor in Arabidopsis plants. At first, we generated independent transgenic lines expressing separate modules of the sensor and subsequently combined the modules by crossing. By this procedure, we came up with the production of two different genotypes, one only bearing the two transcriptional effectors AD-HIF and DBD-pVHL (double over-expressor) and another also encoding a PHD3 expression construct (triple over-expressor). Neither genotype exhibited phenotypic differences when compared to wild-type plants, indicating a very limited effect of the transgenes on the overall plant physiology (Fig. 6A).

Nonetheless, interference of the three transgenes, two of which forming a transcriptional complex, with the regular gene expression profile of the plant could not be ruled out in principle. Therefore, we looked for possible alterations at the transcriptome scale by microarray profiling of the double and triple over-expressors. By this, we aimed at detecting interactions between the synthetic transcriptional complex and endogenous genomic sequences of Arabidopsis. A side consideration of the experiment was that the discovery of genomic targets could supply candidate endogenous reporters for sensor activity, overcoming the need for a fourth transgene encoding for a reporter module, such as the one used in the transient expression experiments described above.

The two genotypes exhibited a broad transcriptome overlap (Supplemental Dataset S1), coherently with the absence of visible phenotypic differences between them. The analysis returned a small set of nine genes that were weakly up-regulated in the triple over-expressor seedlings (Table 1), with a maximum induction in the range of 2 folds. Down-regulated genes were also found (Supplemental File S1) but not taken into account, since they are unlikely to represent direct targets of an activator complex.

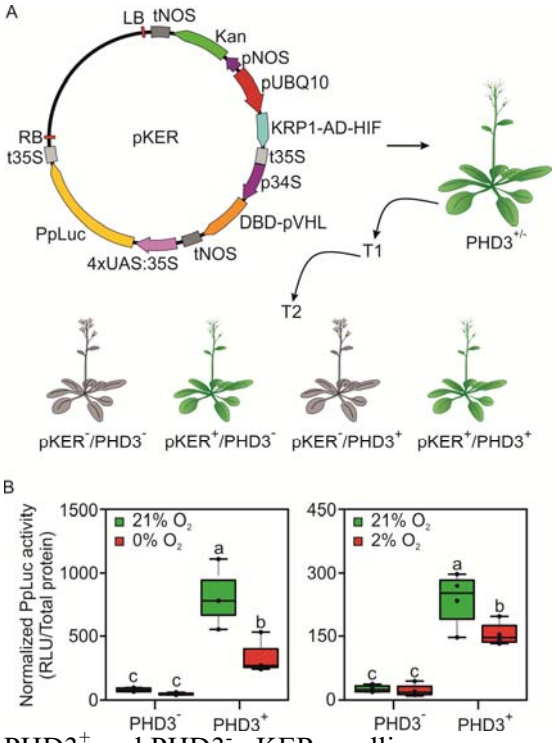
To test whether the differentially expressed genes displayed any oxygen regulation, we analyzed their transcripts in rosette leaves of double and triple over-expressing plants subjected to hypoxia (2% O_2) or maintained under normoxia as a control. We confirmed that all transgenes were properly expressed and found them unaffected by hypoxia (Fig. 6A). However, we could confirm higher expression levels in the triple over-expressors for only one of the nine selected genes, *At1g65390*, which codes for PHLOEM PROTEIN 2 A5 (PP2-A5) (Fig. 6B). Moreover, hypoxia repressed its expression, in the presence of PHD3.

In summary, these data suggest that the transcriptional device based on yeast and mammalian elements, is fully orthogonal to any endogenous pathways converging on transcriptional regulation, which makes it likely to operate without affecting development and growth in plants.



The UAS promoter activity is influenced by oxygen concentration and the presence of PHD3 also in plants

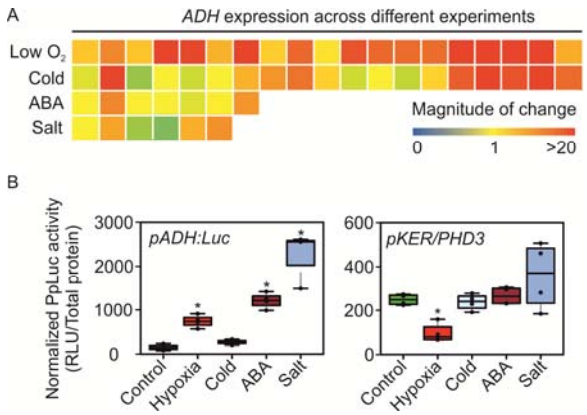
Although one potential endogenous target of the oxygen sensor effectors was found (the *At1g65390* gene), its lower induction in the presence of PHD3 and its possible downregulation by hypoxia make the use of a specifically designed output module still advisable. A further construct, bearing the firefly luciferase gene under the control of the UAS promoter, was therefore delivered into Arabidopsis for stable expression. To reduce the time required for the identification of plants carrying all four transgenes, or specific combinations of them depending on the purpose, we devised to generate multiple expression cassettes. In this new strategy, we incorporated three transcriptional units on a single T-DNA, for the expression of the effector modules (encoding $KRP1_{21\text{-}30}$-AD-$HIF_{554\text{-}576}$ and DBD-$pVHL_{63\text{-}157}$) and an output module expressing the luciferase enzyme (Fig. 7A). Since the *UAS:FLuc* reporter construct used for transient assays in protoplasts did not produce a detectable luciferase signal (Supplemental Fig. S3), we designed an alternative reporter consisting of four tandem repeats of UAS inserted between the A1 and B5 domains of the 35S *CaMV* promoter (Mazarei et al., 2008). The pKER T-DNA (for Kanamycin, Effector and Reporter module) was then delivered to heterozygous Arabidopsis plants carrying the PHD3 sensory module. The integration of three transgenes in a single locus enabled us to rapidly identify plants expressing the transcriptional components of the sensor, whereas the $PHD3^-$ control genotype was recovered thanks to the independent segregation of the PHD3-containing T-DNA (Fig. 7A). High transgene expression was measured in the lines selected for the subsequent experiments (Supplemental Fig. S4).



PHD3$^+$ and PHD3$^-$ pKER seedlings were grown on liquid media and incubated under aerobic (21% O_2 V/V) or hypoxic atmosphere (2% O_2 V/V) for 12 h. After the treatment, total proteins were extracted, quantified and subjected to luminescence quantification. PHD3$^+$ aerobic and hypoxia treated seedlings showed higher luciferase activity than PHD3$^-$ seedlings, indicating that the transcriptional complex is forming and inducing transcription of the reporter gene. Moreover, consistent and significant reduction of reporter activity was detected in hypoxic seedlings coming from plants carrying all the components, while no inhibition was observed in plants devoid of PHD3 (Fig. 7B). These data indicate that the device we examined in the transient test system retained full functionality when stably encoded in Arabidopsis plants.

The HIF-pVHL device exhibits higher hypoxia specificity compared to marker genes

The occurrence of hypoxic conditions in plant tissues can be inferred on the basis of high expression of hypoxia-inducible transcripts, such as *ALCOHOL DEHYDROGENASE (ADH)* (Paul et al., 2001; Licausi et al., 2011b; Gravot et al., 2016). However, an extensive survey of the changes in *ADH* mRNA under different environmental conditions revealed that this gene is actually controlled by many cues, including cold, high salinity and abscisic acid (ABA) (Fig. 8A, Supplemental File S2). We therefore tested whether the synthetic HIF-pVHL based device responds more specifically to low oxygen conditions than an *ADH* promoter-based reporter. To this end, we compared the luminescent output of *pKER/PHD3* seedlings with that of plants at the same developmental stage bearing the firefly luciferase gene driven by the genomic region upstream of



412 the *ADH* gene of Arabidopsis. This *pADH:Luc* transcriptional reporter showed higher activity in
413 response to 10 µM ABA or 150 mM NaCl than after exposure to hypoxia (1% O_2) for 12 h, while
414 low temperature treatment of equal duration did not significantly stimulate luciferase accumulation
415 (Fig. 8B). On the other hand, *pKER/PHD3* seedlings only produced a significant output under
416 hypoxia (Fig. 8B), demonstrating the superior specificity of the synthetic device over a plant
417 promoter-based reporter.
418
419

Discussion

In the present work, we successfully exploited the oxygen-dependent interaction of the mammalian pVHL β-domain with the HIF-CODD to develop a molecular switch to drive gene expression in an oxygen-dependent manner in plant cells and whole seedlings (Fig. 3 and 7). This represents a valuable resource to control metabolism, growth and development under low or high oxygen conditions.

Due to the absence of an efficient transport system for oxygen, its levels vary greatly in plant tissues, depending on environmental parameters and endogenous cues (Sasidharan and Voesenek, 2015). Indeed, photosynthetic release of oxygen from water molecules and its consumption by mitochondrial respiration contribute to its concentration while light, temperature and, especially, exogenous oxygen availability, impact both processes. To adjust cell metabolism, tissue growth and developmental programs to the actual oxygen availability, plants have developed a series of direct and indirect sensing mechanisms of the oxic status. The mechanisms identified so far are tightly interconnected with other signaling pathways, including light perception, sugar sensing, nitric oxide and reactive oxygen species responses. Moreover, the transcriptional network activated in this way has been shown to control a plethora of responses including induction of fermentation, inhibition of chlorophyll biosynthesis, defense against pathogens, and root and shoot development (Giuntoli et al., 2018).

For these reasons, genetic intervention to selectively modulate a subset of these processes in response to variations in oxygen availability represents a true challenge in plants, even accounting for the possibility of precise genome editing offered by the emerging technologies. A valuable alternative is represented by synthetic sensory modules affected by oxygen but not by other cellular pathways (orthogonal), engineered to specifically control one or more processes. The HIF-pVHL device used here has already been proven to work in a heterologous chassis by Alcaide-German et al. (2008), who developed an *in vivo* assay for the study of PHD biochemistry in *S. cerevisiae*. Using a similar set of protein fusions between the pVHL β-domain and the HIF1α-CODD with the DBD and AD of the Gal4 transcription factor, respectively, we confirmed PHD3 activity and selectivity on HIF-CODD, together with the ability of the pVHL β-domain to interact with the hydroxylated form of HIF-CODD (HIF_{Hyp}) also in plant cells (Fig. 2B). Moreover, since no activation of the downstream reporter was observed in the absence of PHD3 (Fig. 2B), we concluded that the interaction of the two chimeric proteins is orthogonal to the host plant system. Indeed, we showed that our synthetic oxygen sensor responds more specifically to hypoxia than simple transcriptional reporters (Fig. 8B and C).

A first main challenge encountered during the optimization of the sensor consisted in expanding the extent of repression brought about by hypoxia (i.e., its dynamic range). Ideally, the device is expected to switch from an on-state under normoxia to an off-state under near-anoxia, passing through a series of gradual states at intermediate oxygen levels. Indeed, this kind of continuous state transition has been observed for the endogenous HIF and ERF-VII systems in animals and plants, respectively (Ceradini et al., 2004; Kosmacz et al., 2015). These oxygen-sensing mechanisms are both based on proteasomal degradation and additionally integrated in a complex network of feed-back and feed-forward loops (Masson et al., 2012; Weits et al., 2014; Kietzmann et al., 2016) that likely ensure stepwise regulation. Relying on a single oxygen-dependent mechanism, instead, our luminescent reporter exhibited only marginal repression when environmental anoxia was imposed (Fig. 2C). By enhancing the turnover rate of the AD-HIF module via a KRP1-degron, we successfully achieved greater response by our oxygen sensor (Fig. 3).

Rational substitution of amino acids crucial for the HIF-pVHL interaction did not lead to any overall improvement of the dynamic range of our sensor. Among all mutations tested, only a Tyr98Asn conversion on the pVHL fragment was associated to mildly (by 10%) increased luciferase activity between anoxia and normoxia, although at the same time decreasing reporter expression under aerobic conditions (Fig. 4D). Nonetheless, this approach allowed the unprecedented identification of a set of pVHL variants (Tyr98Phe, Tyr98Cys, Ser111Ala) whose binding to the HIF1α-CODD was not inhibited by anoxia (Fig. 4C and D, Supplemental Fig. S2C). Future mutational strategies may be directed at displacing residues involved in weak interactions, to modify the local conformation of the complex. An alternative strategy aimed at further reducing reporter expression under anoxia may consist in providing the DBD-pVHL with a repressor domain that is masked by the interaction with AD-HIF, thereby stimulating the competition between repressive VHL monomers and activating heterodimers in an oxygen-dependent manner.

A second limiting aspect of the proposed oxygen sensor is represented by the timescale required to observe substantial output changes after a reduction in oxygen availability. In both transiently and stably transformed cells, 12 hours were necessary to observe a significant decrease in luminescence (Fig. 5A and B), a timeframe unsuitable to monitor oxygen dynamics within tissues on a daily basis. Attainment of the inactive state was comparable with that caused by translation inhibition and thereby in line with the time required for luciferase protein turnover, whereas more rapid activation by reoxygenation occurred in 1 h (Fig. 5B), compatibly with the time required for *de novo* luciferase synthesis. Under this perspective, future improvements of this kind of transcriptional sensor are likely to be achieved with alternative reporters characterized by rapid turnover; for instance, a luciferase fusion with the $KRP1_{21-30}$ destabilization domain could be adopted.

Finally, a third crucial property of a molecular sensor is its sensitivity to the concentration of the analyte. In the case of our device, sensitivity is expected to be determined by PHD affinity for oxygen. *In vitro* assays revealed an apparent K_m of 230-250 μM for HIF-PHDs (Flashman et al., 2008), low enough to be compatible with a sensing function in animal cells; indeed, in human cell cultures, PHD2 activity on a HIF1$\alpha_{556-574}$ peptide was observed to decrease upon a transition from 210 to 100 μM dissolved oxygen and drop in the range of 30-80 μM, with a half-maximum activity close to 50 μM O_2 (Berchner-Pfannschmidt et al., 2008). No *in vivo* kinetic data have, instead, been produced so far for the PHD3 isoform. When expressed transiently in Arabidopsis protoplasts, our PHD3-based sensor worked in response to a moderate decrease in oxygen concentration (5% O_2 V/V, corresponding to 67 μM dissolved O_2) (Fig. 3E), compatibly with PHD2 behaviour. However, the response could not be pushed further at lower oxygen concentrations, at least in the same timeframe. The inability to discriminate signals in the range of 0-5% O_2 currently prevents the exploitation of this molecular switch to drive selective responses to severe oxygen deficiency, such as those arising in plant organs experiencing dark submergence stresses (Lee et al., 2011). Nonetheless, our device holds the potential to detect softer hypoxic conditions, such as the onset of endogenous oxygen gradients that generate inside plant tissues during growth and development (van Dongen and Licausi, 2015).

After correcting some pitfalls that currently limit the applicability of the molecular switch, multiple options for future exploitation can be envisioned. First, as just stated, it can be used to report the actual oxygen status of cells and tissues in plants. This requires equipping the device with a suitable reporter protein characterized by rapid synthesis and turnover, as well as independency from the provision of exogenous substrates. To this end, fluorescent proteins are probably the preferable option, as demonstrated by their employment to build a plethora of synthetic sensors (Walia et al., 2018). Second, the PHD-dependent HIF-pVHL dimerization can be exploited to reconstitute transcription factors different from yeast Gal4, or even enzymes, and drive signaling, metabolic or developmental processes in an oxygen dependent manner in plants. This can be exploited to improve crop yield under challenging oxygen limitation, such as flooding or waterlogging. Alternatively, artificial alteration of oxygen provision to plants or cell cultures can be used, through the HIF-pVHL switch, as a means to control biochemical pathways towards the production of specific molecules.

In summary, with this work we demonstrated that the mammalian oxygen sensing machinery can be harnessed to generate an orthogonal switch able to regulate gene expression in response to hypoxic conditions. It represents the first step for the introduction and enhancement of adaptive traits in response to hypoxic conditions, such as flooding and waterlogging.

Materials and methods

Plant materials and growth conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild-type background in all the experiments. Transgenic plants expressing the individual modules or the entire synthetic regulatory device were obtained by *Agrobacterium*-mediated transformation, applying the floral-dip method (Clough and Bent, 1998). Transgenic seedlings were selected for resistance on the appropriate antibiotic or herbicide and subsequently verified by PCR for the presence of the desired transgenes.

For plant growth in soil, seeds were sown directly on a peat:perlite 3:1 mixture, vernalized at 4°C for 48 h in the dark and then germinated at 22°C day/18°C night with a photoperiod of 12 h. For plant growth in sterility, seeds were sterilized using sequential washes in 70% (V/V) ethanol and 10% (V/V) commercial bleach solution and then rinsed 5 times with sterile distilled water. Seeds were then either sown on liquid or agarized (8 g l^{-1}) MS half-strength medium, vernalized and grown as described above.

Abiotic treatments

The plant material was subjected to low-oxygen atmospheres under darkness, using a hypoxic chamber glove box (COY Lab Products), for the time specified in the text. Abscisic acid (ABA) and NaCl were supplemented in the sterile medium at a final concentration of 10 μM and 150 mM, respectively. Cold stress was applied by incubating at 4°C in the dark. All treatments applied to seedlings lasted 12 h.

DNA fragment cloning and assembly

Transcriptional units were either cloned from genomic DNA, cDNA or *de novo* synthesized by GeneArt (Thermo-Fisher Scientific). Protein-coding sequences were codon optimized using the EMBOSS Backtranseq online tools (McWilliam et al., 2013). The KRP1-AD-HIF transcriptional unit was realized by overlapping PCR using KRP1-AD-Fw and KRP1-FW in combination with HIF-Rv primers. The DBD-pVHL transcriptional unit was realized by overlapping PCR using DBD-Fw, DBD-pVHL-Fw, DBD-pVHL-Rv and pVHL-Rv primers. Four tandem repeats of the UAS element were inserted, via overlapping PCR, 90 bp upstream of the transcriptional start unit of a 464 bp long *CaMV* 35S promoter using 35S-Fw, 35S-UAS-Rv, 35S-UAS-Fw, UAS-35S-Fw, UAS-35S-Rv and UAS-Rv primers. A full list of synthetic sequences used in this work is provided

in Supplemental Information S1. The various DNA fragments were initially sub-cloned either in the pENTR/D-TOPO vector, to be recombined into destination vectors using Gateway LR Clonase II Enzyme mix (Thermo-Fisher Scientific), or in the pCR2.1-TOPO-TA vector (Thermo-Fisher Scientific), to be inserted into expression vectors via a restriction-ligation strategy. The resulting plasmids were then verified using restriction enzymes and, when needed, by sequencing. Plasmid maps were generated with Serial Cloner 2.6.1 (Serial basic) A full list and description of expression plasmids is provided Supplemental Table S1.

The pKER plasmid containing three expression cassettes was obtained by assembling two promoter-terminator cassettes into a compatible SacI restriction site of the pKGWL7 plasmid (Karimi et al., 2002). A first cassette consisted of the Arabidopsis UBQ10 promoter (Grefen et al., 2010), flanked by the SacI and BstBI restriction sites, followed by the 35S *CaMV* terminator (Hirt et al., 1990), cloned in between the SfaAI and PacI recognition sequences. The second cassette comprised the viral FMV 34S promoter (Daubert and Goodman, 1990), separated from the NOS terminator (Depicker et al., 1982) by sequential AfeI and XmaJ1 restriction sites. The two sites present between the promoters and terminators were used to insert specific coding sequences. Specifically, BstBI and SfaAI were used to ligate the KRP1-AD-HIF effector in the first cassette and AfeI and XmaJ1 were used to insert the DBD-pVHL effector in the second cassette; these restriction extremities were incorporated in the primers used to subclone each CDS (BstbIKRP1-Fw and SfaAIKRP1-Rv primers for KRP1-AD-HIF and AfeIDBD-Fw and XmajDBD-Rv primers for DBD-pVHL). The resulting destination vector was finally recombined with an entry vector containing the 4xUAS:min35S sequence to generate the final expression vector pKER4xUAS, whose sequence is provided in Supplemental Information S1. The 5' region upstream of the ADH gene (*At1g77120*) was PCR amplified using Phusion polymerase (Thermo-Fisher Scientific) on Arabidopsis Col-0 genomic DNA and cloned into pENTR/D-TOPO vector (Thermo-Fisher Scientific). Subsequently, the ADH promoter was recombined into pH7LWG by LR clonase (Thermo-Fisher Scientific). A full list of primers used in cloning procedures is provided in Supplemental Table S2. pVHL and HIF site directed mutagenesis were performed by PCR. A full list of primers utilized for site directed mutagenesis is provided in Supplemental Table S3.

Large Scale plasmid DNA purification

Highly concentrated plasmid DNA, required for protoplast transformation, was extracted from a 100 ml bacterial culture (LB medium supplemented with the appropriate antibiotic) by resuspension in 2 ml of a buffer containing 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA (pH 8), lysis in 3 ml of a buffer containing 200 mM sodium hydroxide and 1% (w/v) SDS, followed by neutralization

with 4 ml of ice-cold 3 M potassium acetate in 11.5% (V/V) glacial acetic acid. Nucleic acids were precipitated with an equal volume of isopropanol and plasmid DNA was further purified by RNAse A (Sigma-Aldrich) treatment for 30' at 37°C followed by PEG precipitation (13% PEG 8000 dissolved in 1.6 M NaCl), phenol:chlorophorm extraction and ethanol precipitation. Plasmid DNA was finally eluted in a suitable volume of nuclease-free water to achieve a concentration of 1-2 µg μl^{-1}.

Protoplast isolation and transformation

Protoplast isolation and transformation were performed as reported by Wu et al. (2009) with some modifications. All solutions, with the exception of PEG, were filter sterilized before use. Leaves of three week old plants were deprived of the lower epidermis using paper tape and immerged in an enzymatic solution (0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$, 1% w/v cellulase and 0.4% w/v macerozyme) and incubated at 22°C in the dark for 3 h. The released protoplasts were filtered and washed in W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES). For transfection, protoplasts were centrifuged at 100 g for 1 min and resuspended in MMG (0.4 M mannitol, 15 mM $MgCl_2$, 5 mM MES) to a final concentration of 5 x 10^5 cells ml^{-1}. Then, 100 µl of protoplasts were mixed with 4 µg of each effector and reporter plasmids, plus an equal volume of freshly prepared solution of 40% w/v PEG 4000, 100 mM $CaCl_2$ and 200 mM mannitol. The mixture was incubated for 20 min at room temperature in the dark. After incubation, 440 µl W5 were added and protoplasts were gently pelleted at 100 g for 1 min and resuspended in 1 ml WI (500 mM mannitol, 4 mM MES, 20 mM KCl), Protoplasts were incubated in six-well plates at 22°C in dark for a minimum time of 12 h before starting any treatment. Cycloheximide treatment was performed as reported by Giuntoli et al. 2017 (Giuntoli et al., 2017). For luciferase activity assays, protoplasts were collected and centrifuged at 700 g for 2 minutes. WI was removed, and protoplasts were flash-frozen in liquid nitrogen.

Luciferase activity quantification

Frozen protoplasts were lysed using 50 µl Passive Lysis Buffer (Promega). Firefly and renilla luciferase activities were measured using the Dual-luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Luciferase activity in transgenic seedlings was measured with the same method in total protein pools extracted with Passive Lysis Buffer. Total proteins were quantified using the Bradford protein assay (Bio-rad).

Confocal imaging

Confocal investigations were performed using a Zeiss AiryScan confocal microscope. GFP fluorescence was excited at 488 nm and collected with a 497-554 nm long-pass emission filter. Chlorophyll autofluorescence was excited at 633 nm and collected at 650–750 nm.

Images were analyzed with the ZEN 2010 software (Zeiss). Nuclei were stained with 1µg μl^{-1} 4',6-diamidino-2-phenylidone (DAPI, Sigma-Aldrich), and fluorescence was excited at 405 nm and collected at 410-470 nm.

Molecular dynamic simulations and MM-GBSA

Classical molecular dynamics (MD) simulations were performed with the Amber14 package. The HIF1α-pVHL complex structure was extracted from the crystal structure of a hydroxylated HIF1α peptide bound to the pVHL/elongin-C/elongin-B complex (pdb code: 1LQB, (Hon et al., 2002)).

The water-solvated system was minimized, heated up to 300K and equilibrated by applying positional harmonic restraints (2.0 KCal $mol^{-1}Å^{-2}$) on the protein residues. Finally, a production run of 35 ns in the NPT ensemble was conducted without any restraints. Technical details about the MD simulation are reported in the Supplemental Methods S1. Molecular Mechanics / Generalized Born Surface Area (MM/GBSA) calculations (Kollman et al., 2000; Onufriev et al., 2000; Wang and Xu, 2006; Hou et al., 2011) were performed to determine the binding free energy (ΔG) for each of the considered mutants. We employed the *MMPBSA*.py module of AmberTools (Miller et al., 2012). MM/GBSA calculations were run along short (1 ns) MD runs performed with strong positional restraints (25 KCal $mol^{-1}Å^{-2}$) on all the protein residues outside a 20 Å radius from the Hyp564 residue. This allows focussing on the contribution from the protein residues close to the binding pocket while neglecting the contributions from conformational changes in the flexible region of the protein. In detail, fifteen uncorrelated frames every 1 ns were extracted, the protein fragments were mutated maintaining the coordinates of the wild type backbone and the unchanged sidechain atoms, subsequently the geometry of the mutated residue was relaxed with 1000 optimization steps, keeping the rest of the system frozen, and finally a short MD run was performed to extract 100 frames while skipping the first 10 ps to allow the system to equilibrate. For each frame, MM/GBSA calculations were carried out to obtain the free energy of HIF1α, pVHL and the HIF1α-pVHL complex, and to compute the binding free energy (further technical details are provided in Supplemental Methods S1).

RNA extraction and gene expression analysis

For microarray analysis, pools of 20 seedlings were harvested at the end of the day and total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma). Hybridization against the

Arabidopsis Gene 1.0 ST Array and scanning procedures were performed by Atlas Biolabs GmbH. The raw microarray data were normalized and signal intensities were calculated using RobinA (Lohse et al., 2012) applying the Robust MultiArray (RMA) methodology. Differential expression analysis was carried out using the Limma R package (Ritchie et al., 2015).

For qPCR analysis, total RNA was extracted from 4-week old plants as described by Kosmacz et al. (2015). cDNA was synthesized from 1 µg total RNA using the Maxima Reverse Transcriptase kit (Life Technologies). Quantitative PCR amplification was performed on 12.5 ng cDNA with the ABI Prism 7300 sequence detection system (Applied Biosystems), using the PowerUp SYBR Green Master Mix (Applied Biosystems). Ubiquitin10 (*At4g053290*) was exploited as the housekeeping gene. A full list of the primers used for qPCR is provided in Supplemental Table S4.

Statistical analysis

Ordinary two-way and one-way analysis of variance (ANOVA) and multiple comparisons for statistical differences were performed with GraphPad Prism 7 for Windows 10.

Accession numbers

Microarray data are deposited in the Gene Expression Omnibus Database with the series number GSE118364.

Supplemental Information

Supplemental Figure S1. Comparison of sensor output between the stable and destabilized AD-HIF variants in Arabidopsis protoplasts.

Supplemental Figure S2. *In Silico* prediction and testing of pVHL variants.

Supplemental Figure S3. Basal activity of the exogenous 4xUAS promoter in plants.

Supplemental Figure 4. Over-expression of sensor modules in pKER plants.

Supplemental Information S1. Nucleotide sequences of the constructs used in the study.

Supplemental Table S1. List of plasmids generated in the frame of the study.

Supplemental Table S2. List of oligonucleotides used for cloning.

Supplemental Table S3. List of oligonucleotides used for site directed mutagenesis.

Supplemental Table S4. List of qPCR primers used in this study.

Supplemental Method S1. Extended description of the MD simulation procedure.

Supplemental Method S2. Extended description of the MM/GBSA calculations.

692 **Supplemental File S1.** Microarray comparison of transgenic seedlings expressing two effector
693 modules in the presence or absence of the PHD3 sensory module.
694 **Supplemental File S2.** Changes in ADH mRNA levels in response to different treatments.
695
696
697 **Acknowledgements**
698 We are grateful to Daan A. Weits and Vinay Shukla for technical support with the confocal
699 microscopy and to Giacomo Novi for plant growth supervision.
700
701
702 **Tables**
703 **Table 1. Up-regulated genes in Arabidopsis plants encoding mammalian oxygen sensor**
704 **components.** Fold change values (normalized expression ratios) were calculated from a microarray
705 experiment comparing 7 day-old seedlings bearing both the effector and sensory modules (triple
706 over-expressors) with seedlings only bearing the effector ones (double over-expressors).
707

AGI code	Gene name	Fold change
At1g0220	*NAC003*	0.97
At3g30720	*QQS*	1.12
At1g27730	*Zat10*	1.14
At5g41080	*GDPD2*	1.37
At1g65390	*PP2-A5*	1.39
At1g12400	*TFB5*	1.44
At1g72520	*Lox4*	1.47
At1g80840	*WRKY40*	1.57
At1g11280		1.91

708
709
710 **Figure Legends**
711 **Figure 1.** Sub-cellular localization of the components of the oxygen sensing machinery in
712 Arabidopsis protoplasts. AD-HIF (Gal4 AD fusion of the HIF1α CODD domain), DBD-pVHL
713 (Gal4 DBD fusion of the pVHL β domain) and PHD3 DNA sequences were fused in frame with an
714 N-terminal GFP fluorescent protein sequence, expressed in Arabidopsis mesophyll protoplasts and
715 imaged with a confocal microscope. The signal from the GFP channel is visualised in green. DAPI

staining (1 μg ml^{-1}) marks the nuclei (blue). In the merged image, the red colour is associated to chloroplast auto-fluorescence. Scale bar = 10 μM.

Figure 2. 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 oxygen concentration. Human HIF1-α and pVHL fragments (HIF-CODD and β-domain) were fused in frame with the Gal4 activation domain (AD) or DNA binding domain (DBD) respectively. In the presence of oxygen, AD-HIF is hydroxylated by PHD3 and dimerizes with DBD-pVHL to induce the expression of a firefly luciferase reporter (PpLuc) controlled by a 4xUAS promoter. B, Sensor output in Arabidopsis protoplasts, subjected to 18 hour-long anoxia (0% O_2) or normoxia (21% O_2 V/V) 12 hours after transfection with plasmids bearing the modules depicted in A. Firefly luciferase activity was normalized to that of a renilla luciferase, co-expressed under the control of a constitutive 35S *CaMV* promoter. The average aerobic value produced by the full device (equipped with reporter, sensory and effector modules) was set to 100%. 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%). In the box plots, dots represent single data points, the black line marks the median and the box indicates the interquartile range (IQR). Whiskers extend to data points that are lower than 1.5xIQR away from the box extremities. Different letters indicate statistically significant differences ($p \leq 0.05$) calculated from two-way ANOVA followed by Tukey's post-test.

Figure 3. Engineering of a destabilized version of the AD-HIF protein. A, To increase HIF-CODD turnover, the AD-HIF effector module was modified with an additional destabilizing domain ($KRP1_{21-30}$), promoting its recycling through the 26S proteasome. For the sake of simplicity, the Gal4 domains and the reporter construct are not shown in the schematics. B, Sensor output in an Arabidopsis protoplast transiently transformed with reporter, sensory and effector plasmids and subjected to 18 h anoxia 12 h after transfection. C, Structure of the three known human PHD isoforms, according to the information related to the Uniprot accessions Q96KS0 (PHD1), Q9GZT9 (PHD2) and S5Q9G2 (PHD3). NES stands for Nuclear Export Signal and NLS for Nuclear Localization Signal. D, Sensor output in protoplasts transfected with either one of the three known human PHD isoforms. Effector and reporter modules were equally provided in all transformations. E, Arabidopsis protoplasts were incubated under a range of oxygen concentrations, spanning from complete anoxia to aerobic levels. In the box plots, dots represent single data points, the black line marks the median and the box indicates the interquartile range (IQR). Whiskers extend to data

points that are lower than 1.5xIQR away from the box extremities. Different letters indicate statistically significant differences ($p \leq 0.05$) among means, as assessed from two-way ANOVA (in B and D) or one-way ANOVA (in E) followed by Tukey's post-test to correct for multiple comparisons.

Figure 4. Rational mutagenesis of sensor components to increase its responsiveness to anoxia. A, Crystal structure of HIF1α-CODD and pVHL (Hon et al., 2002). The HIF1α-CODD peptide and its residues are shown in cyan, the pVHL β-domain and its residues in orange, while the α-domain is shown in grey. The oval encloses a magnified view of the hydrogen bond network established between HIF1α-CODD and pVHL residues. Atomic names are given according to the PDB nomenclature. B, Molecular dynamic simulation of the interactions between HIF_{Hyp} and pVHL His115, Ser111 and Tyr98. Hydrogen bond probability involving the donor-acceptor pairs HD1Hyp-ND1His115 are shown in green, HG1Ser111-OD1Hyp in red and HHTyr98-OHyp in blue. The black dotted line represents the hydrogen bond probability for all three bonds to be present. C, MM-GBSA calculations of interaction stability between wild type HIF1α-CODD residue 564 and pVHL residues in the case of Tyr98Asn, Tyr98Phe and Ser111Ala mutations. Data are expressed as binding free energy (ΔG binding). Different letters indicate statistical differences ($p \leq 0.05$) calculated by two-way ANOVA followed by Tukey's post-test (n=15). D, Effect of individual pVHL point mutations on luciferase trans-activation in Arabidopsis protoplasts. The average aerobic output for the wild-type pVHL peptide version (Tyr98) was set to 100%. In the box plots, dots represent single data points, the black line marks the median and the box indicates the interquartile range (IQR). Whiskers extend to data points that are lower than 1.5xIQR away from the box extremities. Different letters indicate statistical differences ($p \leq 0.05$) calculated from two-way ANOVA followed by Tukey's post-test.

Figure 5. Characterization of sensor dynamics. A, Comparison of oxygen sensor output under aerobic conditions (21% O_2 + mock), in the absence of external oxygen (0% O_2 + mock) or in the case of protein synthesis inhibition by 100 μM cycloheximide (21% O_2 + CHX) in Arabidopsis protoplasts. Absolute luciferase activity was monitored at 6, 12 and 18 h after the onset of each condition. B, Sensor output dynamics under reversible oxygen inputs. 12 h anoxia-treated protoplasts were transferred back to aerobic conditions for 1, 3 or 6 h (blue line) and the output was compared with that produced by cells kept under continuous anoxia (red line) or fully aerated conditions (green line) for the same time span. The luminescence output was normalized to the signal corresponding to the initial time point, set to 100%. Dotted lines show ±SD (n=4) and

784 different letters indicate statistical differences ($p \leq 0.05$) between treatments at each time point, as
785 assessed by one-way ANOVA followed by Tukey's post-test .
786
787 **Figure 6.** Molecular and phenotypic consequences of the expression of the synthetic device. A,
788 Phenotypical analysis of two, three and four week-old Arabidopsis plants overexpressing KRP1-
789 AD-HIF and DBD-pVHL in the absence ("double") or presence ("triple") of PHD3. Bars equal 1
790 cm. B, Absolute mRNA levels for the three transgenes (*KRP1-AD-HIF*, *DBD-pVHL* and *PHD3*)
791 assessed by qPCR in 3 week-old plants expressing two (double) or three (triple) modules and
792 subjected to aerobic (21% V/V O_2) or hypoxic conditions (2% V/V O_2) for 12 h. C, qPCR
793 assessment of expression levels of the genes identified as induced by the microarray analysis (Table
794 1 and Supplemental File S1) in double or triple transgenic plants, under aerobic or hypoxic
795 conditions (2% V/V O_2). In the box plots, dots represent single data points, the black line marks the
796 median and the box indicates the interquartile range (IQR). Whiskers extend to data points that are
797 lower than 1.5xIQR away from the box extremities. Different letters indicate statistical differences
798 ($p \leq 0.05$) calculated from two-way ANOVA followed by Tukey's post-test.
799
800 **Figure 7**. Responsiveness of the synthetic O_2-sensor stably expressed in Arabidopsis. A, Map of the
801 T-DNA used to link the KRP1-AD-HIF, DBD-pVHL and 4xUAS:35S:Luc expression cassettes.
802 The construct was transformed into heterozygous *35S:PHD3* plants ($PHD3^{+/-}$) and first generation
803 transgenic plants (T1) were selected on kanamycin for the presence of pKER (in the hemizygous
804 state). From their offspring (T2), two genotypes of interest were isolated upon the independent
805 segregation of the PHD3- and pKER-containing T-DNAs: *pKER* ($pKER^{+}/PHD3^{-}$) and *pKER/PHD*
806 ($pKER^{+}/PHD3^{+}$), corresponding to the colored Arabidopsis plants. Sensor performance in one of
807 the T2 populations produced is shown. B, Normalized firefly activity in *pKER* seedlings, stably
808 expressing the effector components of the oxygen responsive device, in the presence or absence of
809 PHD3. Sensor output was recorded after 12 h treatments under complete anoxia (left) or 2% O_2 V/V
810 (right). In the box plots, dots represent single data points, the black line marks the median and the
811 box indicates the interquartile range (IQR). Whiskers extend to data points that are lower than
812 1.5xIQR away from the box extremities. Different letters indicate statistically significant
813 differences among means ($p \leq 0.05$) calculated from two-way ANOVA followed by Tukey's post-
814 test.
815
816 **Figure 8.** Comparative response of *pADH:Luc* and PHD3-positive pKER seedlings to abiotic
817 stresses. A, Changes in *ADH* mRNA across different experiments, gathered with the aid of the

Genevestigator reference expression database (Hruz et al., 2008) and listed in Supplemental File S2. B, Luminescent output of *pKER/PHD3 pADH:Luc* seedlings in response to four different abiotic treatments. In the box plots, dots represent single data points, the black line marks the median and the box indicates the interquartile range (IQR). Whiskers extend to data points that are lower than 1.5xIQR away from the box extremities. Asterisks indicate statistically significant differences ($p \leq 0.05$) from the mean of the control, calculated from one-way ANOVA comparison followed by Fisher's LSD test.

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