Methods

Multiparametric real-time sensing of cytosolic physiology links hypoxia responses to mitochondrial electron transport

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Summary

- Hypoxia regularly occurs during plant development and can be induced by the environment through, for example, flooding.
- To understand how plant tissue physiology responds to progressing oxygen restriction, we aimed to monitor subcellular physiology in real time and in vivo. We establish a fluorescent protein sensor-based system for multiparametric monitoring of dynamic changes in subcellular physiology of living Arabidopsis thaliana leaves and exemplify its applicability for hypoxia stress.
- By monitoring cytosolic dynamics of magnesium adenosine 5'-triphosphate, free calcium ion concentration, pH, NAD redox status, and glutathione redox status in parallel, linked to transcriptional and metabolic responses, we generate an integrated picture of the physiological response to progressing hypoxia. We show that the physiological changes are surprisingly robust, even when plant carbon status is modified, as achieved by sucrose feeding or extended night. Inhibition of the mitochondrial respiratory chain causes dynamics of cytosolic physiology that are remarkably similar to those under oxygen depletion, highlighting mitochondrial electron transport as a key determinant of the cellular consequences of hypoxia beyond the organelle.
- A broadly applicable system for parallel in vivo sensing of plant stress physiology is established to map out the physiological context under which both mitochondrial retrograde signalling and low oxygen signalling occur, indicating shared upstream stimuli.

Introduction

Oxygen (O2) availability is essential for plant life. Although mitochondrial respiration typically dominates O2 consumption rates, other vital processes, such as oxidative protein folding, biosynthetic metabolism, and xenobiotic detoxification, require O2 as a substrate (Malmström, 1982; Tu & Weissman, 2004; Raymond & Segre, 2006). Plant tissues can feature a large range of O2 concentrations, from above atmospheric O2 content, as shown by photosynthesis, to bona fide anoxia (Borisjuk et al., 2007; Borisjuk & Rolletschek, 2009; van Dongen & Licausi, 2015). Hypoxic conditions not only occur regularly in various tissues; they also play an important regulatory role in development by, for example, determining meristem function (Considine et al., 2017; Signorelli et al., 2018; Weits et al., 2019) and germ cell fate (Kellie & Walbot, 2012). However, in many instances, hypoxia acts as a stressor that requires acclimation and may limit plant growth and productivity. Waterlogging of roots is a particularly common situation, but stems and leaves are also prone to hypoxia at high-water flooding, especially in the absence of active photosynthesis. Major metabolic re-orchestration (van Dongen et al., 2009; Rocha et al., 2010; Sweetlove et al., 2010; Mustroph et al., 2014), hormonal regulation (Fukao et al., 2006; Xu et al., 2006; Fukao & Bailey-Serres, 2008), and reprogramming of gene expression (Mustroph et al., 2010; Licausi et al., 2011b) act to circumvent hypoxia or to alleviate its impact on cell and tissue physiology and have been studied in depth. Ubiquitous responses of cell physiology include a decreased adenylye
energy charge and cytosolic acidification (Roberts et al., 1984; Ratcliffe, 1999; Geigenberger et al., 2000; Rolletschek et al., 2002; Felle, 2006). Various different mechanistic frameworks have been proposed to account for those changes (Ratcliffe, 1999; Felle, 2001). However, an integrated understanding of the dynamics in subcellular physiology has not yet been established and several questions, such as the mechanistic origin of the cytosolic acidification and the reactive O2 species (ROS) production during hypoxia, remain controversial (Clanton, 2007; Pucciariello et al., 2012; Gonzali et al., 2015). A number of mechanistic steps involved in endogenous O2 signalling of plant cells could be elucidated (Gibbs et al., 2011; Licausi et al., 2011b; Weits et al., 2014; Kosmacz et al., 2015), which have evolved independently from the O2-sensing pathways in animals (van Dongen & Licausi, 2015), even though common components were identified recently (Masson et al., 2019). The activity of different pathways and different O2 perception mechanisms in plants has been proposed in plants (Holdsworth, 2017), and a comprehensive picture is yet to emerge. Interestingly, the role of the mitochondrial or oxidative phosphorylation in O2 monitoring and signalling remains diffuse. Conceptually, it would appear favourable to monitor O2 availability in association with respiration as the major cellular process that is sensitive to fluctuations in O2 supply, and often also responsible for O2 depletion.

Based on the observation that the transcriptomic responses to mitochondrial electron transport inhibition and to low O2 stress show considerable overlap, we have recently hypothesised that both conditions may trigger similar physiological consequences (Schmidt et al., 2018b; Wagner et al., 2018). Pharmacological inhibition of mitochondrial electron transport, mainly by the Complex III inhibitor antimycin A (AA), has been the basis for the field of mitochondrial retrograde signalling (Schwarzländer et al., 2012a; Umbach et al., 2012; Ng et al., 2013a), with recent breakthrough discoveries of several molecular players involved (De Clercq et al., 2013; Ng et al., 2013b, 2014; Shapiguzov et al., 2019). Yet, remarkably little is known about the cell physiological changes that are induced as a direct consequence of respiratory inhibition. Such an understanding will be critical to pinpoint upstream stimuli and endogenous sensing mechanisms for mitochondrial dysfunction and to further test the hypothesis of common players involved in O2 signalling and mitochondrial retrograde signalling (Schwarzländer & Finkemeier, 2013; Finkemeier & Schwarzländer, 2017).

A technical limitation to understand the regulatory role of O2 partial pressures in living tissues has been the reliance on O2-sensitive electrodes, which cannot be straightforwardly scaled to finer tissue resolution, or individual cells and cell compartments (Schmidt et al., 2018b). Over recent years, a number of fluorescent protein sensors have become available with favourable technical properties for live sensing of a growing set of cofactors, small ions, and molecules, as well as physical parameters. Several of the biosensors have been successfully introduced into plants and have delivered novel insights into the dynamics of subcellular physiology in vivo (Schwarzländer et al., 2016; Grossmann et al., 2018; Hilleary et al., 2018; Walia et al., 2018). A particularly attractive methodological avenue has been multiplexing of sensors to measure two or more parameters simultaneously. Sensor multiplexing in plants has been achieved in only a few cases, in which typically two sensors of different colour were used in the same cells and tissues. As a result, it has been possible to discriminate calcium ion (Ca2+) dynamics in two different subcellular compartments of the same cells (Loro et al., 2012), to monitor changes in the electrical and the pH gradient in single mitochondria (Schwarzländer et al., 2012b), and Ca2+ and abscisic acid in the cytosol (Waadt et al., 2017), to draw conclusions about the biological role of synchronous or asynchronous changes. Despite much future potential, this approach is intrinsically limited by the need to generate plants equipped with multiple sensors, or the same sensor with distinct subcellular localisations. Further, most of the currently available fluorescent protein sensors cover the green part of the fluorescence spectrum, which severely restricts their straightforward combination. New sensors with clearly distinct spectral properties (such as red fluorescence) are required, which are only available and sufficiently well established in specific cases (Tantama et al., 2011; Zhao et al., 2011; Ermakova et al., 2014; Fan et al., 2015), and even fewer have been established in plants (Keinath et al., 2015). Even if all sensors were available in any colour, the feasible spectral range would accommodate a maximum of about three sensors. Those constraints have rendered multiplexing of fluorescent protein sensors in plants a niche application so far, even though efforts date back to the advent of the development of fluorescent proteins for in vivo sensing, and despite the promise of unprecedented biological insights (Walia et al., 2018).

As an alternative approach to sensor multiplexing in the same cells of the same plant, we reasoned that parallelised measurements of different plant sensor lines would circumvent limitations on the number of different sensors, need for distinct sensor colours, and higher order transgenic lines. Knowledge of the dynamic changes in a larger set of physiological parameters (or in a single parameter in different subcellular locations) in vivo promises insights into their interaction and the temporal hierarchy of changes that reflect signals and responses. The principle of capturing whole sets of parameters in parallel has been decisive for the success and analytical power of several ‘omics’ approaches, such as transcriptomics or metabolite profiling. Although the development of analogous ‘physomics’ by in vivo sensing is certainly not yet in reach, even a smaller set of parameters to define the cell physiological consequences of a stimulus has the potential to provide novel and fundamental insight into the relationships between events underpinning plant development, stress responses, and signalling.

Here, we set out to elucidate the cell physiological dynamics induced by low O2 stress. We induce progressive hypoxia in Arabidopsis leaves in a well-defined multit威尔 plate format. We establish parallel live monitoring of five different hypoxia-responsive parameters of cell physiology. Focusing on the cytosol to exemplify the multiparameter approach to subcellular in vivo sensing, we find characteristic and robust dynamics that define the cytosolic hypoxic response in leaves. A comparison of hypoxia with pharmacological inhibition of mitochondrial electron transport highlights mitochondrial respiration as a dominant primary cause of the monitored cell physiological changes under hypoxia.
Materials and Methods

The following procedures are described in Supporting Information Methods S1–S8: chemicals; plant materials and transformation; plate reader settings used for O2 quantification; O2 quantification with the PreSens system; ethanol quantification; transcript quantification; extended night and sucrose treatments; calibration of circularly permuted yellow fluorescent protein (cpYFP) using leaf extracts.

Multiwell plate preparation for plate reader experiments

Transparent 96-well plates (#82.1581; Sarstedt, Nümbrecht, Germany) with a well volume of 400 μl were filled with 300 μl assay medium (10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.8 (potassium hydroxide), 10 mM magnesium chloride, 10 mM calcium chloride, 5 mM potassium chloride). Rosette leaves of 5- to 6-wk-old Arabidopsis plants were trimmed with sharp scissors to 30 ± 1 mg fresh weight (FW) using precision scales. As leaves of different age and different regions of a single leaf can differ in respiration rate (Sew et al., 2015), only single leaves of similar and intermediate age were used to fill individual wells. The trimmed leaf was cut perpendicular to the vein into three pieces of similar size. The leaf pieces were layered on top of each other, carefully rolled using soft tweezers, and submerged in a single well prefilled with assay medium. Once all leaf pieces were prepared, their intercellular air spaces were infiltrated by placing the plate in a desiccator, applying vacuum for 3 min, and releasing the vacuum over c. 5 min. Then each well was topped up to the rim with assay medium (not degassed). All the aforementioned steps were performed in ambient light and at room temperature. The plate was then inserted into a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) pre-equilibrated to 25°C in the dark. In the absence of photosynthetic effects and to equilibrate the medium with ambient air O2 levels. To induce hypoxia, wells were sealed with an airtight transparent film under minimal light conditions and control wells were left open. The plate was reinserted into the plate reader and kept at 25°C in the dark. In the absence of photosynthetic O2 evolution, we anticipated a net consumption of O2 due to respiration activity of the leaves in all wells. Whereas open wells can be continuously resupplied with atmospheric O2, the O2 content in sealed wells was expected to decrease gradually. O2 was quantified using the O2-sensitive dye MitoXpress Xtra (Agilent Technologies, Santa Clara, CA, USA) (Fig. 1b). When measured by time-resolved fluorescence, the dye allows stable readings over a wide range of O2 concentrations, where increasing fluorescence lifetimes indicate decreasing O2 levels with a maximal lifetime of c. 68 μs (manufacturer’s note). The O2 concentration in open wells remained almost stable over the course of the experiment, suggesting that an equilibrium between consumption and resupply from ambient air is already established in the preincubation phase between application of the vacuum, to deplete the leaf intercellular spaces from air, and start of fluorescence reading. By contrast, the dye lifetime rapidly increased when wells with leaf material were sealed (Fig. 1b(j)). Notably, the lifetime in open wells with leaf material started slightly below that of control wells without leaf material, indicating that initial O2 consumption exceeds the resupply with atmospheric O2. Conversion of the lifetime values using calibrated atmospheric O2 concentrations (which must be regarded an approximation) suggested a drop below 1% of atmospheric O2 levels in sealed...

Results

A multiwell plate setup standardises progressive O2 depletion in Arabidopsis leaf tissue

To establish standardised hypoxic conditions in Arabidopsis in a synchronised fashion, rosette leaves of 5- to 6-wk-old plants were trimmed to the same FW and submerged in assay medium on a 96-well plate (Fig. 1a). Vacuum infiltration was used to remove residual air from intercellular leaf spaces to avoid variable O2 reserves between samples. Wells were topped up to the rim with nondegassed assay medium and the plate was preincubated in a plate reader in the dark for 30–60 min to avoid residual photosynthetic effects and to equilibrate the medium with ambient air O2 levels. To induce hypoxia, wells were sealed with an airtight transparent film under minimal light conditions and control wells were left open. The plate was reinserted into the plate reader and kept at 25°C in the dark. In the absence of photosynthetic O2 evolution, we anticipated a net consumption of O2 due to respiration activity of the leaves in all wells. Whereas open wells can be continuously resupplied with atmospheric O2, the O2 content in sealed wells was expected to decrease gradually. Fluorescence was read sequentially for each well using the ‘well multichromatics’ mode. Appropriate excitation (Ex) and emission (Em) wavelengths were chosen for each sensor: ATeam and YC3.6: Ex = 435 ± 10 nm/Em = 485 ± 10 and 540 ± 7.5 nm; Peroxo-mCherry: Ex = 400 ± 5 and 570 ± 10 nm/Em = 530 ± 20 and 610 ± 5 nm; cpYFP, reduction–oxidation-sensitive green fluorescent protein 2 (roGFP2), and pH-GFP: Ex = 400 ± 5 and 482 ± 8 nm/Em = 530 ± 20 nm. The ‘orbital averaging’ mode was used to distribute the 70 excitation light flashes per reading cycle and well on a circle with a 4 mm radius. This allows fluorescence emissions read after each excitation flash to be averaged to minimise the contribution of local fluorescence artefacts when measuring structured samples such as Arabidopsis leaf pieces.
wells within 2 h after applying the airtight film (Fig. 1b). Orthogonally, we quantified O₂ with a fiber-optic O₂ meter system (PreSens, Regensburg, Germany) (Fig. 1c). Leaf pieces were prepared as already described (Fig. 1a), but the wells were sealed with plasticine to allow insertion of a glass fiber that, together with a sensor film, allows calibrated O₂ readings. These data...
independently validated our fluorescence measurement and indicated a rapid decline in O₂ concentration in the presence of living leaf tissue after restricting atmospheric O₂ supply, albeit at a slightly lower rate (compare Fig. 1b(ii),c). Although the assay setup to induce leaf hypoxia comes with several technical characteristics that differ from a naturally occurring scenario, a key strength lies in the gradual (as opposed to abrupt) decrease of O₂ concentrations as established by tissue respiration itself. Such a gradual transition is a likely characteristic of most naturally occurring situations of environmentally induced O₂ stress.

Transcript and metabolite responses define the onset of hypoxia stress

To test whether the O₂ restriction leads to a hypoxic response in the rosette leaf tissue, we quantified transcripts that had previously been shown to be increased in leaves of Arabidopsis plants shifted to hypoxic or anoxic conditions (Lee et al., 2011; Licausi et al., 2011b). Established marker transcripts (ALCOHOL DEHYDROGENASE1 (ADH1), PYRUVATE DECARBOXYLASE1, PLANT CYSTEINE OXIDASE1, SUCROSE SYNTHASE1, PHYTOGLOBIN (previously referred to as HEMOGLOBIN1) and HYPOXIA-INDUCED UNKNOWN PROTEIN7) were evaluated at all analysed time points after sealing (1, 3, 6 and 12 h), and this behaviour was overall robust between experiments (Figs 1d, S1a). By contrast, these transcripts in open wells were modestly increased 1 h into the experiment but then decreased in abundance. The transcript changes validate that a hypoxic response is mounted under the assay conditions. We next measured ethanol levels in sealed and open wells as an indicator of hypoxia-induced metabolic rearrangement (Fig. 1e; Licausi et al., 2010). Whereas 1 h after sealing the ethanol levels in sealed and open wells were still comparably low, pronounced differences occurred after 3 h. Open wells showed minor but noticeable ethanol production within the first 3 h. Ethanol concentration was c. 2.5–3.5 times higher in the sealed samples and progressively increased within the 12 h of measurement (Figs 1e, S1b).

Setting up five different fluorescent biosensors to monitor cytosolic physiology by multiwell multiplexing

To investigate the physiological impact of low O₂ stress, we optimised the use of genetically encoded fluorescent protein sensors (Grossmann et al., 2018; Hilleary et al., 2018; Walia et al., 2018). We selected five sensors that cover key physiological parameters specifically in the cytosol: magnesium adenosine 5’-triphosphate (MgATP₂⁻) through ATTeam1.03-nD/nA (De Col et al., 2017), free Ca²⁺ through YC3.6 (Krebs et al., 2012; Loro et al., 2012, 2016), pH through cpYFP (Schwarzländer et al., 2014; Behera et al., 2018), and the glutathione redox potential $E_{GSSH}$ through Grx1-roGFP2 (Marty et al., 2009). NADH : NAD⁺ had not been accessible for live monitoring in plants, which is why we generated lines for the fluorescent NAD redox sensor Peredox-MCherry (Hung et al., 2011) to be included in the multiplexing approach. Overlapping spectral properties of the underlying fluorescent proteins prevent parallel readout of these sensors in a single plant (Fig. 2a). Parallelised multiwell plate-based fluorimetric measurements, however, allow for a synchronised induction of hypoxia in a large number of different genetic backgrounds (i.e. different sensor lines) and replicates side-by-side, offering an alternative approach to sensor multiplexing within a single experiment. All selected sensors show ratiometric behaviour, which provides internal normalisation of measurements independently of sensor protein abundance (Hilleary et al., 2018). The operational principles of the selected sensors are outlined in Fig. 2a and Notes S1. To run the biosensor assays, two sensor fluorescence excitation or emission channels were each measured and background corrected using fluorescence signal from plant material lacking any sensor that was recorded in parallel for each experiment. The ratio between both values was log₁₀-transformed to accurately represent the ratio variance and restore symmetry of the ratio data and normalised to start at zero (Fig. 2b). The intensity changes of both individual channels relative to each other hold information about the technical soundness of the ratio change and require inspection as a measure of quality control (Fig. 2c). This inspection is relevant in particular for measurements at low O₂ concentrations as the maturation of the protein chromophores is O₂ dependent (Heim et al., 1994; Inouye & Tsuji, 1994). Whereas no artefacts are expected in single chromophore sensors given that nonmature protein will not fluoresce, this can in principle cause interference for two fluorophore sensors. Further, changes in autofluorescence from the leaf material over time need to be controlled for by background correction. Representative fluorescence intensities before background correction and the corresponding fluorescence signal from sensor-free leaf material used to produce data shown in Fig. 2 highlight the importance to use material with and without protein sensor in parallel (Fig. S2a). In particular material excited at 400 nm exhibits noticeable stress-induced autofluorescence, which can be faithfully removed by the correction.

Progressive O₂ depletion induces pronounced physiological dynamics in the cytosol of Arabidopsis leaf cells

We next applied the multiplex biosensing approach to the hypoxia assay making use of the five Arabidopsis lines with cytosolic biosensor expression. None of the recordings from the open well controls showed obvious changes over the course of 12 h (Fig. 2b), indicating that sole submergence in well-aerated medium (Fig. 1b(ii)) was not sufficient to result in major responses in the measured parameters. By contrast, restricted O₂ resupply in the sealed wells induced pronounced physiological dynamics that were reliably reproducible and different for each of the five sensors (Fig. 2b). Since comparable dynamic signatures have not been available so far, they were carefully and individually assessed for their technical validity, also drawing on well-established information about the cell physiological changes during low O₂ stress.

Cytosolic MgATP²⁻ levels decreased quickly, with data from sealed and open wells differing statistically from 48 min onwards (cyt-ATTeam 1.03-nD/nA in Fig. 2b). Despite the speed of the
response, the measurement cycle time (> 10 min) was sufficiently small to resolve the dynamics (Fig. 2b) but can be adjusted to much lower cycle times at the cost of sample numbers per plate.

Apparent MgATP$^{2-}$ levels reached a plateau after c. 4 h, before they further declined. The individual cyan fluorescent protein (CFP) and YFP emission channels showed strictly inverse behaviour for the first 8 h, indicating a bona fide Förster resonance energy transfer (FRET) response (Fig. 2c). A pronounced drop in MgATP$^{2-}$ concentrations is consistent with decreased energy charge and cytosolic acidification, which destabilises the MgATP$^{2-}$ complex. The additional YFP : CFP ratio decrease between 8 and 12 h, by contrast, is mainly due to an increase in...
CFP emission, which cannot be unambiguously attributed to a decrease in cytosolic MgATP$_2^-$ concentration.

Cytosolic free Ca$_{2+}$ levels remained unchanged in sealed wells for much longer than MgATP$_2^-$, starting to statistically differ from those in open wells after 120 min (NES-YC3.6 in Fig. 2b). Free Ca$_{2+}$ concentrations then showed a high increase to reach a maximum at c. 6–8 h. This indicates Ca$_{2+}$ release from the Ca$_{2+}$ stores into the cytosol, which has also been observed by other methods and proposed to act as a fundamental mechanism of acclimation to low O$_2$ (Igamberdiev & Hill, 2018). Like for ATeam, the individual CFP and YFP channels of YC3.6 showed strictly inverse dynamics for the first 8 h, indicating a bona fide FRET response (Fig. 2c). Since the subsequent decrease in YFP:CFP ratio was dominated by an increase in CFP emission, which cannot be accounted for by a FRET response, this late time window should not be interpreted as Ca$_{2+}$ clearance from the cytosol.

The cytosolic NADH:NAD$^+$ ratio increased particularly quickly, reaching significant differences between sealed and open wells after 24 min (cyt-Peredox-mCherry in Fig. 2b). After c. 4 h, the NAD pool started to be reoxidised and returned to its starting redox state after c. 12 h. A rapid increase of cytosolic NADH:NAD$^+$ ratio is consistent with an accumulation of metabolic reductant before fermentation is induced (Licausi et al., 2010). The emission of NAD-insensitive mCherry remained almost constant for 8 h and even increased slightly between 8 and 12 h when the NAD-sensitive T-Sapphire signal decreased (Fig. 2c), indicating a faithful dependence of the T-Sapphire:mCherry ratio on NAD redox state over the full course of the experiment.

A drop in cytosolic pH also started early, and the sensor readouts of the open and sealed wells started to turn statistically different after 48 min (cyt-cpYFP in Fig. 2b). The reported pH indicated that the cytosol increasingly acidified with the progression of the hypoxic treatment. The initial drop was dominated by a decrease in emission from 482 nm excitation, whereas the apparent acidification from c. 6 h was driven by an emission increase when cpYFP is excited at 400 nm (Fig. 2c). This behaviour of the two channels is consistent with the in vivo response of the sensor to pH (Schwarzländer et al., 2014), indicating faithful measurement of pH dynamics through background subtraction of stress-induced autofluorescence (Fig. S2a). We independently validated the degree of cytosolic acidification through pH-GFP (Moseyko & Feldman, 2001). A comparison with calibrated intracellular pH values obtained from cpYFP- and pH-GFP-expressing Arabidopsis seedling roots (Fendrych et al., 2014; Behera et al., 2018) suggests that the cytosolic pH at 12 h drops below 6 (Fig. S3a). To also empirically estimate the absolute pH response for the specific measurements in leaf tissue and the experimental conditions of the multiwell setup, we optimised a calibration regime for the cpYFP sensor, based on leaf extracts of 5- to 6-wk-old rosettes (Fig. 3b–i). Cytosolic pH was c. 7.5 before hypoxia treatments and in the controls, and it dropped by c. 1.5 pH units under hypoxia to reach a pH$^*$ of c. 6.0.

The cytosolic glutathione redox potential $E_{GSH}$ remained unchanged immediately after sealing but afterwards increased and became statistically different from that of open wells after 180 min (cyt-Grx1-roGFP2 in Fig. 2b). After a very strong increase, a plateau was reached after 10 h. The degree of Grx1-roGFP2 oxidation was amongst the strongest so far observed at any stress in the plant cytosol (Schwarzländer et al., 2016) and is likely to reflect the maximum value that is technically feasible, as oxidation of leaf pieces with 100 mM H$_2$O$_2$ did not cause any further ratio increase (Fig. S5j). Since the redox state of the glutathione pool is set by the balance between electron influx from metabolically derived NADPH and electron efflux (to reduce H$_2$O$_2$, for example), as well as by the total glutathione pool size, the measured oxidation suggests a major reset in this balance in response to hypoxia. The emission after excitation at 400 and 482 nm shows strict counter-parallel behaviour over the course of 12 h, validating that the sensor response reports a very strong oxidation response of the cytosolic glutathione pool (Fig. 2c).

The data in Figs 1 and 2 collectively validate the rapid induction of low O$_2$ stress in Arabidopsis leaves using the multiwell setup, while enabling simultaneous monitoring of the physiological responses by online in vivo sensing. The setup is highly versatile. For instance, we used either less plant tissue mass (25 mg instead of 30 mg; compare Fig. S4a(i,ii), to slow down the depletion of available O$_2$ per well, or plant tissue that had not been vacuum infiltrated (Fig. S4a(i)), to increase the available O$_2$ content per well and to maintain the biological variability of intercellular space volumes. In both cases, the hypoxic response at the physiological level is significantly delayed, and lack of vacuum infiltration further increased the variability between individual wells. The robustness of the setup was confirmed throughout a large succession of independent experiments, as exemplified in Fig. S4(b). We observed similar sensor dynamics when we used 1-wk-old intact Arabidopsis seedlings instead of leaves from adult plants (Fig. S4c,d). Despite analogous response patterns, the responses were delayed, consistent with only 4.5 mg (instead of 30 mg) biomass per well. This suggests that the sensors monitor a fundamental physiological response to low O$_2$ stress that is not restricted to a particular plant tissue or developmental stage.

Increasing stored O$_2$ delays the onset of physiological dynamics

To further test if the observed changes were really due to O$_2$ depletion, we replaced the assay medium in the wells with perfluorodecalin (PFD), a fluorocarbon liquid that is biologically and chemically inert, has favourable optical properties, and is able to dissolve c. 80-fold more O$_2$ than water can (49 ml vs 0.63 ml O$_2$ per 100 ml at 25°C; Littlejohn et al., 2010, 2014). Our rationale was that PFD would increase the amount of available O$_2$ in a sealed well and delay hypoxia responses. The sensors in sealed wells overall behaved similar to what we had observed in aqueous assay medium, but the onset of responses was consistently shifted by c. 7–8 h for all sensors (Fig. 3a,b). For instance, instead of immediately dropping, the MgATP$_2^-$ levels remained almost stable for c. 8 h. Then they dropped sharply to a local minimum within 4 h. The kinetics of the drop were comparable to those observed in aqueous medium, and the relative order of the onset
of the five different responses was strictly preserved (Fig. 3a). Though the major increase in the NADH : NAD$^+$ ratio was also delayed by c. 7 h, it also occurred more gradually and its subsequent recovery occurred with similar kinetics to that in aqueous medium, indicating that a hypoxic response to induce metabolic rearrangement was also effectively activated in PFD.

Consistently, the hypoxia marker transcripts in sealed wells did not show a delayed increase in abundance, and the majority peaked at 12 h. This suggests that the transcriptional markers respond earlier to decreased O$_2$ availability (i.e. to milder deviations from normoxia) than cytosolic physiology as monitored by the biosensors (Figs 3c, S5).
Physiological responses to progressing hypoxia in the cytosol are robust at modified carbon supply

A lack of O₂ rapidly restricts ATP production through the mitochondrial respiratory chain that requires O₂ as the terminal electron acceptor in oxidative phosphorylation. Plants alleviate the energy crisis by activating glycolytic processes that produce ATP from sugar breakdown (Narsai et al., 2011; Antonio et al., 2016). Accordingly, Arabidopsis plants that are unable to degrade starch to fuel glycolysis are more susceptible towards hypoxia, whereas external supply of sucrose increases hypoxia resistance (Loreti et al., 2018). To elucidate the impact of carbon (C) availability on the dynamics of cytosolic physiology to low O₂ stress, we prepared leaf pieces from plants after an extended night for depletion of starch-based C stores, or fed leaf pieces with sucrose to increase and sustain C availability. None of the recorded physiological parameters showed obvious and reproducible differences as a result of interference with C availability compared with the controls when wells were sealed (Fig. 4). Cytosolic Ca²⁺ seemed to behave differently in experiments employing either extended night or sucrose treatments. The lack of clear reproducible differences between treated and control plants suggest, however, that this may more likely be explained by experimental variation instead of C status. Noticeably, sucrose feeding led to a reduction of the NAD pool in open wells, but other clear impacts of the treatments were not observed (Fig. S6). The lack of an impact of C availability on the set of physiological dynamics investigated is unexpected, but it emphasises their robustness and suggests active regulation instead of passive, demand-driven metabolic responses.

Pharmacological inhibition of the mitochondrial respiratory chain leads to similar changes in cytosolic physiology as hypoxia

The mitochondrion is the primary cellular consumer of O₂, rendering its functions particularly sensitive to the impact of hypoxia. This raises the question as to what extent changes in mitochondrial respiratory activity account for the dynamic changes in cytosolic physiology that occur during hypoxia. We have recently found that the transcriptomic response to hypoxia shows remarkable similarities to transcriptomic changes in response to pharmacological inhibition of the mitochondrial electron transport chain (mETC; Wagner et al., 2018), opening up a potential role of mitochondrial retrograde signalling in hypoxia responses, which may even share common upstream stimuli. Yet, the impact on physiological status as directly affected by both conditions had remained hard to assess. We thus aimed for a direct comparison of the response to mETC inhibition in cytosolic physiology. Instead of restricting the O₂ supply, we kept wells open to avoid any decrease in O₂ and treated the leaves with 100 μM AA, a potent inhibitor of mETC Complex III.
preventing electron flux to Complex IV as a reduction site for molecular O₂. Remarkably, the response of cytosolic physiology to pharmacological inhibition of mitochondrial electron transport without limiting O₂ supply resembled the response of cytosolic physiology under O₂ depletion qualitatively to a striking extent. Treatment with AA led to a decrease in cytosolic MgATP²⁻ levels, an increase in cytosolic Ca²⁺, a transient reduction of the NAD pool, an acidification of the cytosol, and an oxidation of the glutathione pool (Fig. 5a). This indicated that inhibition of electron transport activity is sufficient to account for the hypoxia-associated physiological changes downstream. However, most parameters changed less severely than under progressing hypoxia (Figs 2b, 5a). We hypothesised that a major difference between removal of O₂ as a respiratory substrate and inhibition of the cytochrome c pathway of the mETC may be the activity of plant alternative oxidase (AOX). AOX bypasses two proton translocation sites in the cytochrome pathway (Complexes III and IV) and thereby uncouples respiration from ATP production. AOX can be pharmacologically inhibited through salicylhydroxamic acid (SHAM) or n-octyl gallate (nOG), and we tested both inhibitors in combination with AA separately. Using the decline in cytosolic MgATP²⁻ as a marker, the combinatorial treatments of AA with either SHAM or nOG consistently suggested a strong resemblance to O₂ depletion (Fig. S7). We verified the additive inhibitory effect of AA and SHAM on respiration in an O₂ consumption assay (Fig. 6a). Whereas leaf pieces treated exclusively with AA were still able to consume O₂, a combined treatment with SHAM suppressed respiratory activity (Fig. 6a). The combined AA+SHAM treatment was thus extended to leaf pieces from all sensor lines (Fig. 6b). Remarkably, the dynamics of all five assessed parameters were nearly identical between AA+SHAM treatment and progressing hypoxia (Fig. S8). This resemblance strongly suggests that impairment of mitochondrial electron transport in the presence of O₂ is sufficient to account for major hypoxia-induced rearrangements in cytosolic physiology.

Discussion

Multiparameter fluorescent biosensing as a versatile technology in plant stress

A growing number of genetically encoded protein sensors have been successfully established for use in plants (Grossmann et al., 2018), but overlapping spectral properties prevent simultaneous use in a single plant to explore the interplay of physiological changes and signalling events. Parallelised analysis allows monitoring of a variety of parameters in multiple replicates almost simultaneously. Integration of replicate data results in a robustness that allows drawing conclusions about the interaction of parameters assessed in parallel and the temporal sequence of their changes. Further, sampling from a running live sensing experiment for other analyses is possible, which extends the range of measured parameters side by side and allows correlation between cellular events, as exemplified here for O₂, metabolite, and transcript measurements. Though similar routes have been taken for cultured yeast and mammalian cells (Morgan et al., 2011; Birk et al., 2013; Zou et al., 2018), fluorescence plate readers have...
rarely been used in combination with intact plant tissues (see Rosenwasser et al., 2010, 2011 for important exceptions), and not with multiple biosensor proteins in parallel. Potential optical interference, such as chlorophyll autofluorescence, can be effectively circumvented or corrected for, as we recently demonstrated for ATeam and roGFP2 (De Col et al., 2017; Voon et al., 2018; Nietzel et al., 2019). YC3.6 has analogous spectral properties to ATeam and cpYFP to roGFP2.

Though heterogeneous responses between cell compartments, cells, and tissues are best studied by confocal microscopy (Behera et al., 2018), the plate reader approach allows for higher throughput in parallel experiments as well as time series from the range of seconds to hours or even days.

Limitations come from the need of a sufficiently strong sensor signal for a meaningful signal-to-noise ratio and the requirement that the dynamic range of the sensor needs to match the measured parameter in the individual compartment. Further, although the sensors employed here are largely inert to physiological pH changes in the cytosol (except from the pH sensor cpYFP) (Nagai et al., 2004; Schwarzländer et al., 2008, 2014; Imamura et al., 2009; Albrecht et al., 2011; Hung et al., 2011; De Col et al., 2017; Hartmann et al., 2018), particularly acidic cellular compartments lead to quenching of the sensor signal. Sensors of different affinities and acidity-inert sensors need to be selected in those cases to match the individual local conditions.
Establishing multiparameter biosensing during progressing hypoxia in leaves

Here, we exemplify in vivo biosensor multiplexing by monitoring five parameters of cytosolic physiological dynamics in response to hypoxia stress. A simple, well-standardised protocol was established to gradually, but quickly, induce low O₂ stress in Arabidopsis leaves. As the experiments are performed in the dark (except for the excitation flashes that are necessary to obtain a readout), no significant O₂ evolution through photosynthesis is expected and O₂ concentrations gradually decrease in sealed wells due to the respiratory activity of the tissue. This gradual decrease in O₂ resembles a scenario that is naturally meaningful, and which is conceivable to occur in plant leaves flooded in the night or by muddy water. The O₂ concentration in sealed wells using 30 mg leaf material dropped rapidly, reaching levels of < 1% a few hours after sealing the wells. The gradual nature of the O₂ depletion, as well as the ability to flexibly control its rate by varying the tissue amount or the well volume, offers an advantage in the interpretation of the physiological response when compared with hypoxia or anoxia treatment regimes in which O₂ concentrations are changed abruptly. Though the setup faithfully establishes differences in O₂ dynamics between low O₂ treatments and controls, the option to also assess nonsubmerged samples will require future optimisation to control for optical differences as a result of the presence or absence of an immersion liquid.

A specific issue to consider under hypoxia is that maturation of sensor chromophores requires O₂ and is unlikely to occur under strong hypoxia (Tsien, 1998). De novo synthesised sensor proteins will then not fluoresce, which would not affect the reading, but slightly different maturation kinetics between two chromophores within the same sensor (e.g. T-Sapphire and mCherry in the Peredox-mCherry sensor) could result in artefactual ratio changes, and this has indeed been observed in fast-growing cells (Hartmann et al., 2018). Similar effects may occur by selective degradation of one of the chromophores. However, the relative dynamics of the two individual channels per sensor suggest that these issues play no major role in this setup and that the sensors faithfully respond to their respective parameters. An exception is the late responses of the two FRET sensors (ATeam and YC3.6) after ~8 h, when an increase in CFP signal occurs that is not matched by a decrease in the YFP signal, suggesting a technical artefact. Further interference of the hypoxic treatments with the sensing approach could, in principle, be caused by the cytosolic acidification. For all other parameters, sensor variants with low pH sensitivity were used to minimise potential artefacts. The sensor dynamics appeared independent in timing from the acidification kinetics, and the initial response of the Peredox-mCherry sensor was inverse to what would be expected from an acidification-induced artefact, providing strong evidence that pH changes did not affect the analysis to any major extent. Having carefully considered potential shortcomings, as well as known hypoxia-induced physiological changes, such as cytosolic ATP depletion and acidification, we conclude that the multiparameter fluorescent sensing approach allowed for meaningful monitoring of hypoxia-associated cell physiology with remarkable reproducibility.

Defining the hypoxic response of cytosolic physiology

We selected the cytosol as a major site of cellular metabolic integration and of molecular transport between cellular compartments. The parallel measurements reveal that NAD redox state, pH, and MgATP₂⁻ respond particularly quickly to declining O₂ availability within the first hour (Fig. 2b). Changes in free Ca²⁺ and EGSH set in much later. The O₂ concentration in sealed wells decreased gradually, but rapidly, reaching 10%, 4%, and 1% after < 1.5 h, < 2.5 h, and < 5 h, respectively (Fig. 1b,c). The drop in MgATP₂⁻ (Fig. 2b) that was noticeable within minutes may indicate proactive regulation already at very mild hypoxia or that local O₂ concentrations at the mETC are significantly lower than those we have quantified in the assay medium. The former is in line with previous observations of metabolic readjustment occurring far above the O₂ threshold that would be biochemically limiting for respiration (Geigenberger et al., 2000; van Dongen et al., 2009). The plateau reached by the ATeam sensor after 4 h is likely to be set by the dynamic range of the sensor (De Col et al., 2017). Cytosolic Ca²⁺ accumulation, which started 2 h after well-sealing and reached a plateau after 6–8 h, is in line with previous observations using the luminescent AEQUORIN probe (Sedbrook et al., 1996). The extended rise in cytosolic free Ca²⁺ may reflect a collapse of ATP-dependent membrane gradients releasing Ca²⁺ from store compartments, such as the extracellular space, the endoplasmic reticulum (ER), or the vacuole. Rise in cytosolic Ca²⁺ concentrations and local generation of signals by combined action of (passive) Ca²⁺ influx through channels and stalling of (active) Ca²⁺ efflux through pumps and cotransporters has been suggested as a central mechanism of acclimation to low O₂ (Igamberdiev & Hill, 2018).

A shift of the NAD pool towards reduction (Fig. 2b) is the earliest of the measured responses in cytosolic physiology to decreasing O₂ levels. Initial NAD reduction is followed by an apparent reoxidation after ~5 h that takes the NAD redox state gradually back to its pre-hypoxic state. A switch to fermentative metabolism, consistent with very early induction of fermentation-associated transcripts (e.g. ADH1 > 3-fold after 1 h) and the increased release of ethanol (Fig. 1d,e), may contribute to the reoxidation, although the exact mechanism is not clear and will require future investigation.

Cytosolic acidification as a hallmark of hypoxic physiology started early and progressed for ~6 h. Cytosolic acidification to pH values of ~6.8 provides optimal conditions for hypoxic metabolism to run, while suppressing aerobic metabolism (Davies et al., 1974; Roberts et al., 1984). Calibration of the sensor responses can introduce serious bias, while the relative ratio changes represent dynamic changes in a meaningful manner (Wagner et al., 2015; De Col et al., 2017). We estimate the magnitude of the cytosolic pH shift nevertheless using a calibration previously performed in Arabidopsis roots (Fig. S3a) and optimise an empirical calibration regime specifically for the experimental setup (Fig. S3b–i). Both approaches consistently suggest a pH drop to ~6.5, which is even more acidic than previous
...estimates by other methods (Gout et al., 2001; Kulichikhin et al., 2008). The acidification mechanism is not fully established. Contributions from lactate accumulation and loss of proton pumping across the plasma membrane and the tonoplast as a result of ATP depletion have been implicated (Felle, 2001; Gout et al., 2001). Correlative evidence for the latter may be drawn from the similar kinetics of MgATP$^{2-}$ depletion and acidification.

$E_{\text{GSH}}$ (Fig. 2b) undergoes a delayed, but surprisingly strong, transition. The entire dynamic sensor range is passed (Fig. S3j), meaning that the plateau that is eventually reached is likely set by the sensor. Given a cytosolic $E_{\text{GSH}}$ of $c. -320$ mV under resting conditions, oxidation appears to go beyond $-240$ mV, reaching the $E_{\text{GSH}}$ range in which the ER typically operates (Schwarzländter et al., 2008, 2016). Similarly, strong stress-induced cytosolic oxidation in Arabidopsis has only been observed by calibration with millimolar concentrations of external $\text{H}_2\text{O}_2$ and by severe depletion of the glutathione pool (Aller et al., 2013). If the oxidation was solely due to the detoxification of ROS, then ROS production would need to be strongly increased under hypoxia. This may appear counterintuitive, as ROS production depends on $\text{O}_2$ tension, which is below 1% when the oxidation rates are highest, but it is not inconceivable given that fully anoxic conditions are unlikely to be established in our system. An alternative explanation is a strong decrease of total cytosolic glutathione concentration, potentially as a result of glutathione turnover and impaired synthesis due to the ATP crisis (Joshi et al., 2019).

The delay of the responses by submergence in $\text{O}_2$-rich PFD validates their specificity to $\text{O}_2$ (Fig. 3a,b). Hypoxia transcript markers were nevertheless, by contrast, triggered early (Fig. 3c). Notably, all hypoxia transcript markers used here are under control of the transcription factor RAP2.12 belonging to subgroup VII ETHYLENE-RESPONSE-FACTORs (Licausi et al., 2011a; Kosmacz et al., 2015). Assuming a key regulatory function, RAP2.12-mediated signalling appears not strictly dependent on the cytosolic physiological rearrangements observed by the biosensors. Although PFD delays $\text{O}_2$ depletion in the well, submergence in PFD still restricts $\text{O}_2$ diffusion compared with air, and this appears to be sufficient to trigger RAP2.12 signalling. Even though MgATP$^{2-}$ changes were minor within the first 8 h (Fig. 3a), adenylate energy charge, which depends on the...

![Diagram](image-url)
individual concentrations of ATP, ADP and AMP, may drop markedly and interact with early signalling, as observed recently (Schmidt et al., 2018a). Submergence in PFD may also induceethylene production, which acts as an early signal in the transition from submergence to mild hypoxia. Ethylene induces core hypoxia genes, and this may happen before major physiological rearrangements occur (van Veen et al., 2013).

The robustness of the physiological changes against manipulation of the C status appears surprising, however. Although C status is a well-established determinant of hypoxia tolerance, our observations suggest that the physiological transitions occur independently (Figs 4, S6). The latter implies that the altered cytosolic physiology provides the environment for hypoxic C metabolism, but C availability itself has little influence on the establishment of this environment.

A prominent role of mitochondrial electron transport in hypoxic cell physiology and signalling

We observed striking similarities in the physiological dynamics of progressing O₂ depletion and mitochondrial dysfunction, as induced by inhibition of Complex III by AA (Fig. 5), and of Complex III and AOX simultaneously by combined AA and SHAM treatment (Fig. 6). Shared rearrangements of cell physiology show that inhibition of mitochondrial electron transport is sufficient to explain the sensor dynamics under progressing hypoxia. Consequently, inhibition of mitochondrial electron transport is likely to be the dominant cause of the cytosolic physiological rearrangements under hypoxia (Fig. 7). This conclusion contributes a new line of evidence to the ongoing debate as to whether mitochondria play a role in O₂ signalling and hypoxia responses (Shingaki-Wells et al., 2014; Schmidt et al., 2018b). It is nevertheless unlikely that mitochondrial electron transport inhibition arises from a simple substrate limitation, since the affinity of cytochrome c oxidase to O₂ is high (Kₐ in the range of 100–150 nM; Hoshi et al., 1993; Millar et al., 1994). Nitric-oxide-mediated inhibition of electron transport and the orchestration of respiratory C metabolism upstream have been found as hallmarks of low O₂ stress (Geigenberger, 2003; Gupta et al., 2011). Both make suitable candidate mechanisms to account for the remarkable similarity of the physiological fingerprints of hypoxia and electron transport inhibition. Finally, several players of retrograde signalling pathways from the mitochondrion to the nucleus have been identified using AA-based screening approaches (Ng et al., 2014). However, what the natural conditions are for which those retrograde pathways have evolved and under which they are actually activated have not been established. The striking overlap in responses at the transcriptional level (Wagner et al., 2018), and cell physiological level (this study), identifies hypoxia as a likely natural condition under which mitochondrial retrograde signalling is activated.

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Author contributions

SW conceived the research together with MS and with specific input from OVA and RRS; SW, RRS, and MS designed the experiments and interpreted the data. SW, JS, PF, SL, ME, TN, and CR optimised the novel methods. SW, JS, PF, SL, and JHMS collected and analysed the data. SW and MS wrote the manuscript with support from JS, OVA, AJM, JTVD, and RRS.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.