The stability and nuclear localization of the transcription factor RAP2.12 are dynamically regulated by oxygen concentration

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ABSTRACT

Plants often experience low oxygen concentrations as the consequence of reduced oxygen availability in their environment or due to a high activity of respiratory metabolism. Recently, an oxygen sensing pathway was described in Arabidopsis thaliana which involves the migration of an ERF transcription factor (RAP2.12) from the plasma membrane to the nucleus upon hypoxia. Moreover, RAP2.12 protein level is controlled through an oxygen-dependent branch of the N-end rule pathway for proteasomal degradation. Inside the nucleus, RAP2.12 induces the expression of genes involved in the adaptation to reduced oxygen availability. In the present study, we describe the oxygen concentration and time-resolved characterization of RAP2.12 activity. A reduction of the oxygen availability to half the concentration in normal air is sufficient to trigger RAP2.12 relocalization into the nucleus, while nuclear accumulation correlates with the first induction of the molecular response to hypoxia. Nuclear presence of RAP2.12 may not only depend on relocalization of existing protein, but involves de novo synthesis of the transcription factor as well. After re-oxygenation of the tissue, degradation of RAP2.12 in the nucleus was observed within 3 h, concomitant with reduction in hypoxia responsive gene transcripts to normoxic levels.

Key-words: Arabidopsis thaliana; anoxia; confocal laser scanning microscopy; Ethylene Response Factor; hypoxia; mEos; N-end rule; oxygen sensing; photoconversion; re-oxygenation.

INTRODUCTION

As aerobic organisms, plants require molecular oxygen to produce ATP via respiration. In their natural environment, plants often experience low oxygen conditions as a consequence of flooding or waterlogging (Bailey-Serres & Voesenek 2008). Moreover, during development and growth, the physical constraints for oxygen diffusion through multiple cell layers as well as increased metabolic activity may cause steep oxygen gradients within specific tissue or cell types (van Dongen et al. 2003; Ho et al. 2010). Unlike animals, no active distribution systems exist in plants to deliver oxygen to internal tissues. Instead, oxygen transport mainly depends on diffusion which can lead to steep oxygen gradients in multilayer plant tissue such as roots (Armstrong et al. 1994; Mancuso & Boselli 2002), seeds (Borisjuk & Rolletschek 2009), anthers (Kelliher & Walbot 2012), fruits (Ho et al. 2010) and tubers (Licausi et al. 2011a).

When oxygen levels drop, aerobic metabolism is reduced with a subsequent reduction in ATP availability. Concomitantly, the metabolism of storage products such as starch, proteins and lipids is reduced (Geigenberger 2003). Moreover, energy-saving pathways are favoured over those that are more expensive in terms of ATP (Bologa et al. 2003). When oxygen becomes limiting for oxidative phosphorylation, ATP production relies on fermentative metabolism. Its activation coincides with the strong up-regulation of the expression of fermentative genes as well as genes encoding various transcription factors and other hypoxia-induced proteins of which no function has been revealed yet (van Dongen et al. 2009). The precise control of these adaptive responses to hypoxia is mediated via a sensing mechanism that involves oxygen-dependent localization of the transcription factor RAP2.12 and its oxygen-dependent degradation via the N-end rule pathway (Gibbs et al. 2011; Licausi et al. 2011b).

The transcription factor RAP2.12 belongs to a group of Ethylene Responsive Factors (ERF-VII group; Nakano et al. 2006), which acts as an activator of the anaerobic response (Licausi et al. 2011b). ERF-VII proteins are characterized by a highly conserved N-terminal motif that, in the presence of oxygen and NO, is recognized as degradation signal submitting the proteins to a branch of the proteasome-dependent N-end rule pathway (Gibbs et al. 2014). Initially, the terminal methionine is cleaved by Met-aminopeptidase when a cysteine residue is positioned at the second position of the N-terminus. Subsequent oxidation of the exposed cysteine amino acid by plant cysteine oxidases (PCOs) triggers the conjugation of an arginylation of the protein to the N-terminus via Arg-tRNA protein transferases (ATEs) (Graciet et al. 2010; Weits et al. 2014). In turn, arginylation of the protein is a signal for E3-ubiquitin ligase (PRT, named after the proteolysis...
process) to poly-ubiquitinate Lys residues to prepare the protein to undergo degradation via the 26S-proteasomal pathway (Varshavsky 2011).

Among the five members that constitute the ERF-VII group in Arabidopsis, RAP2.12 is constitutively expressed in many cell types but, at the same time, degraded in the presence of oxygen (Licausi et al. 2011b). Nevertheless, some RAP2.12 protein was shown to be protected from degradation via sequestration to the plasma membrane, where it binds to ACYL-COA BINDING PROTEIN 1 and 2 (ACBP1 and ACBP2) (Licausi et al. 2011b). Upon hypoxia, GFP-tagged RAP2.12 was shown to shift its localization from the plasma membrane into the nucleus, where it triggers the induction of the hypoxic responsive genes (Licausi et al. 2011b).

A detailed characterization of these processes in terms of oxygen-concentration dependence and reaction time of the relocation was not provided previously. Our earlier studies showed that 4 h of 1% (v/v) oxygen in air triggered RAP2.12:GFP relocation into the nucleus. However, it is expected that this might occur already at higher oxygen concentrations, especially since several other studies reported that low-oxygen response genes are already induced when the oxygen concentration in air is decreased to 10% (v/v) (Loreti et al. 2005; van Dongen et al. 2009). With the present study, we characterize the nuclear accumulation of RAP2.12 protein in a time- and oxygen-concentration-dependent manner by means of confocal laser scanning microscopy and gene expression analyses in leaves of Arabidopsis thaliana. Our results show that not only relocation of existing RAP2.12 protein but probably also de novo synthesis of the transcription factor during hypoxia is involved in the induction of the molecular hypoxic response in plants. Furthermore, we investigated how fast RAP2.12 protein degrades upon re-oxygenation of the tissue.

MATERIALS AND METHODS

Plant material and growth conditions

Fusion protein constructs between RAP2.12 and GFP or mEos were used to visualize the localization of the transcription factor within the epidermal cells of A. thaliana. T4 homozygous transgenic plants overexpressing RAP2.12:GFP and described in Licausi et al. (2011b) were used. 35S:RAP2.12:mEos were generated in this study as described subsequently. Arabidopsis seeds were sown in moist soil, stratified at 4 °C in the dark for 48 h, and germinated at 22 °C day/18 °C night (photoperiod of 8 h light with an illumination of 120–180 μmol m⁻² and 16 h darkness), 60% humidity during the day and 75% at night. Two weeks after germination, seedlings were moved to single pots and maintained in the same growth conditions described above. For all experiments, 5-week-old plants were used.

Low oxygen treatments

Low oxygen [1–21% (v/v) oxygen in air] and re-oxygenation treatments were performed in the absence of light to avoid O₂ production via photosynthesis as described before (Licausi et al. 2011b). At 0800 h, intact plants were placed inside an H85 HypOxistanation (HypOxygen, Frederick, MD, USA) containing an artificial atmosphere consisting of oxygen and nitrogen in the proportions described in the text and figure legends. During the hypoxic and re-oxygenation treatments, plants were maintained in the dark at 22 °C. The time periods of the treatment that was used for various experiments are indicated throughout the text.

Construct and transgenic plant preparation

For 35S:RAP2.12:mEos, the coding sequence of RAP2.12 (At1g53910) was fused to monomeric, photoconvertible fluorescent protein (mEos; Mathur et al. 2010) by overlapping PCR using Phusion High Fidelity DNA Polymerase (Thermo Scientific, Schwerte, Germany) and then introduced into pENTR/D-topo vector (Life Technologies, Darmstadt, Germany) to generate pENTR-RAP2.12:mEos. A list of the primers used for cloning is provided in Supporting Information Table S1. This ENTRY vector was recombined into the destination vector pH7WG2 (Karimi et al. 2002) using the LR reaction mix II (Life Technologies) to obtain the expression vectors 35S:RAP2.12:mEos. Transgenic plants were obtained using the floral dip method (Clough & Bent 1998). T₅ seeds were screened for hygromycin resistance and independent lines expressing the fluorescent fusion protein were selected using confocal laser scanning microscopy.

RNA extraction, cDNA synthesis and real-time qPCR analyses

In order to quantify gene expression, mRNA was isolated and reverse transcribed to cDNA. Total RNA was isolated from leaves using a phenol-chloroform extraction protocol. Briefly, 100 mg (fresh weight) of plant material was homogenized in 600 μL of extraction buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 2% SDS, 5 mM EDTA and 255 μM β-mercapto-ethanol. Next, KCl was added to a final concentration of 321 mM to each sample and then kept on ice for 15 min. Samples were centrifuged at 5000 rcf for 5 min at 4 °C and the supernatant was collected. LiCl was added to a final concentration of 4 M and samples were incubated 2 h at 4 °C. Samples were centrifuged for 20 min at 4 °C at 20 000 rcf and the pellet was resuspended in 400 μL of nuclease-free water containing 0.3 M NaAC. An equal volume of a phenol/chloroform (1:1) mixture was added. Samples were centrifuged for 5 min at 20 000 rcf at room temperature. The aqueous phase was recovered and an equal volume of chloroform was added and mixed. Samples were centrifuged for 5 min at 20 000 rcf at 4 °C. Again, the aqueous phase was recovered and 2.5 volumes of ice-cold absolute ethanol. The samples were kept for 30 min at −20 °C. After centrifugation (10 min at 13,000 g, 4 °C), ethanol was removed and the dry pellet was resuspended in 50 μL of nuclease-free water. Total RNA was subjected to a DNase treatment (TURBO DNase free kit; Ambion, Ambion GmbH, Berlin, Germany) and cDNA was
synthesize using SuperScript III reverse transcriptase kit (Life Technologies), following the manufacturer’s instructions. Quantitative real-time PCR amplification was carried out with the ABI Prism 7900 sequence detection system [Applied Biosystems (Life Technologies), Darmstadt, Germany] using a Power SYBR Green master mix (Life Technologies), as described previously (Licausi et al. 2010). A list of the real-time RT-qPCR primers used in this study is provided in Supporting Information Table S1.

Confocal laser scanning microscopy

For GFP and mEos imaging, leaves from independent stable transformed plants were analysed with a Leica DM6000B/SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). For GFP observations, whole plants were treated with a hypoxic atmosphere as described in the figure legends. Fully developed leaves were subsequently excised with a scalpel and placed onto a microscope slide with a droplet of water to image the epidermal cell layer. In case of microscopic observations after hypoxic treatments, the whole rosette was placed in a hypoxic chamber containing the oxygen atmosphere specified in each figure legend. Leaves were harvested in the hypoxic chamber and dissected further, as described above, in normoxic atmosphere within 20 min after the exposure to normoxia.

When 35S::RAP2.12::mEos plants were used, single leaves were excised and observed under confocal microscope to select those showing a green fluorescence signal, and therefore identified as transgenic. Photoconversion of RAP2.12::mEos protein was carried out by illumination of a pre-selected area Region of Interest (ROI) modus with the ultraviolet (UV) laser (405 nm, 70 mW) in six cycles of 45 s light with a 15 s break (Woźni et al. 2012). Subsequently, the leaf was treated with hypoxia (1% O₂, 4 h) while still mounted on the glass slide, which was not sealed to allow gas exchange. After 4 h, the glass slide with the leaf was rapidly moved from the hypoxic chamber to the confocal microscope. The photoconverted area of the leaf could be easily distinguished due to the shift from green to red fluorescence.

To assess the membrane-to-nucleus relocalization of RAP2.12::GFP, areas of about 20 cells were analysed for the appearance of fluorescent signal in the nucleus. GFP was excited with the 488 nm laser and mEos was excited using the 488 or 561 nm laser wavelength for green or red fluorescence, respectively. Emission of green fluorescence from GFP and mEos was detected between 500 and 520 nm, while red emission from photoconverted mEos was observed between 610 and 630 nm.

Image analysis of the fluorescent intensity was performed using the ImageJ software (public domain software from the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA, http://imagej.nih.gov/). Green and red signal intensities were measured by selecting the membrane or nuclear regions of a cell using the polygon selection option.

Statistical analyses

The differences between mean values between genotypes or treatments were evaluated by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison procedure using SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany). The respective number of replicates and P-values is as indicated in the text.

RESULTS

Time-resolved survey of RAP2.12 localization in the nucleus upon hypoxia exposure

To test how long after the onset of hypoxia it takes before RAP2.12 accumulation in the nucleus can be observed, Arabidopsis plants expressing the fluorescent fusion protein RAP2.12::GFP were exposed to 1% O₂ (v/v) for different periods of time (0, 30, 60, 120, 180 and 240 min) and the intracellular localization of RAP2.12::GFP in epidermal cells was visualized by confocal laser scanning microscopy. In parallel, whole rosettes were harvested to analyse the expression level of typical hypoxia marker genes that possess the RAP2.12 recognition sequence (ATCTA) in their promoter (Licausi et al. 2011b), including ALCOHOL DEHYDROGENASE 1 (ADH1), PYRUVATE DECARBOXYLASE 1 (PDC1), HEMOGLOBIN 1 (HB1), SUCROSE SYNTHASE 1 (SUS1), LOB DOMAIN PROTEIN 41 (LBD41), PLANT CYSTEINE OXYDASE 1 (PCO1) and 2 (PCO2), and the HYPOXIA-RESPONSIVE UNKNOWN PROTEIN HUP9.

Throughout the entire time course of the experiment, membrane-associated RAP2.12::GFP remained visible, while after 3 h of hypoxia nuclear RAP2.12::GFP was detected as well (Fig. 1a,b). The fluorescent signal observed in the chloroplasts results from chlorophyll autofluorescence (Supporting Information Fig. S1). Induction of the hypoxia marker genes was observed much earlier. Their expression increased already within the first 30 min after the treatment was started, while PDC1, LBD41, PCO1, PCO2 and HUP9 reached their expression maximum within 30 min (Fig. 1c). We therefore concluded that accumulation of RAP2.12 to a concentration that is sufficient to be detected by confocal microscopy requires longer than the time needed to induce the expression of the direct target genes of RAP2.12.

Oxygen-concentration-dependent localization of RAP2.12 in the nucleus

The previous experiment showed that RAP2.12::GFP protein needs 3 h at 1% oxygen to accumulate in the nucleus to a detectable level. Next, we characterized at which oxygen concentration RAP2.12::GFP accumulates in the nucleus to initiate the response to hypoxia. For this, A. thaliana plants expressing RAP2.12::GFP were treated with different oxygen concentrations [0, 1, 3, 5, 10, 12.5, 15 and 21% O₂ (v/v) in air] for 4 h. Subsequently, we studied the localization of RAP2.12::GFP protein and collected samples for gene expression analysis. Under aerobic and mild-hypoxic conditions (>10% oxygen), only membrane localization of the
Figure 1. Localization of RAP2.12:GFP and hypoxia responsive gene expression during different time periods with 1% oxygen in air.

(a) Subcellular localization in leaf epidermal cells of stably transformed 35S::RAP2.12:GFP Arabidopsis thaliana plants in normal air (time zero) as well as after 30, 60, 120, 180 and 240 min of 1% hypoxia. The micrographs are representative for at least four biological replicates. Scale bar, 10 μm. (b) Histogram represents the percentage of epidermal cells with nuclear localization of RAP2.12:GFP at different time points after the onset of hypoxia (n = 3). Each micrograph contained 20 cells on average. (c) Differential expression of hypoxia responsive genes in 35S::RAP2.12:GFP plants as compared with air-treated control samples (n = 5). Data represent mean values ± standard deviations. Values marked with different letters are considered to be significantly different according to a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparison procedure (P <= 0.05).
GFP signal was observed (Fig. 2a,b). At 10% oxygen, nuclear accumulation of RAP2.12 became apparent, although this was not uniform throughout the tissue; only half of the cells exhibited GFP signal in the nucleus (Fig. 2b). At oxygen concentrations below 10%, the nuclear GFP signal became stronger with decreasing oxygen concentrations (Fig. 2a,b). When measuring the expression level of a selected set of hypoxia responsive genes, we observed that the induction of some hypoxia marker genes (HB1, ADH1, PDC1, LBD41, PCO1 and PCO2) is initiated by oxygen concentrations below 10%, but it generally becomes significant only at 1% O₂ in air.

**Contribution of protein relocalization and de novo synthesis to the induction of the anaerobic response**

To investigate whether nuclear localization of RAP2.12 upon hypoxic conditions is due to the release of RAP2.12 from the plasma membrane or due to nuclear import of de novo synthesized RAP2.12 during hypoxia, or both, we designed a construct in which RAP2.12 was fused to the N-terminus of the photoconvertible fluorescent protein mEos (35S:RAP2.12:mEos). Expression of this chimeric protein in transgenic Arabidopsis plants confirmed the localization pattern described before for RAP2.12:GFP (Licaisi et al. 2011b; Supporting Information Fig. S2). In particular, under hypoxia, RAP2.12:mEos co-localized with the nuclear dye diamidino-2-phenylindole (DAPI) (Supporting Information Fig. S3). The mEos fusion protein localized at the plasma membrane during aerobic conditions and accumulated in the nucleus after 4 h at 1% O₂ (Fig. 3).

Subsequently, we photoconverted the membrane-localized RAP2.12:mEos protein prior to the hypoxic treatment by a series of high-intensity blue/violet laser light exposure cycles. This converted the already present fusion protein from a green fluorescent protein to a stable red fluorescent protein. Indeed, the ratio of red to green fluorescence in the membrane strongly increased from 0.2 before photoconversion to 1.9 after photoconversion (Fig. 3b). Maintenance of photoconverted 35S:RAP2.12:mEos leaves under normoxic conditions for 4 h did not affect the localization of the red form of RAP2.12:mEos (Supporting Information Fig. S4). Instead, after 4 h hypoxia, red fluorescence was also observed in the nucleus. The occurrence of this red (photoconverted) mEos signal in the nucleus upon hypoxia provides proof that pre-existing protein was translocated from the membrane to the nucleus upon hypoxia. Together with the red fluorescent signal in the nucleus, an equal intensity of green fluorescence was observed in the nucleus during hypoxia (Fig. 3b; red to green ratio in the nucleus upon hypoxia was 1.0). The green nuclear fluorescence can derive from the translocation of non-photoconverted green mEos protein from the membrane into the nucleus but, because the intensity of the green fluorescence as compared with red is much higher in the nucleus than in the plasma membrane, it could also be hypothesized that de novo RAP2.12:mEos synthesis and subsequent translocation to the nucleus occur during hypoxia. Furthermore, it should be noted that after the UV exposure for photoconversion, fluorescence from the chloroplasts also increased (Fig. 3a). However, the same effect was observed when wild-type leaves were exposed to repetitive UV flashes (Supporting Information Fig. S5), and is therefore explained as an artefact due to UV-induced chloroplast damage.

**Time-resolved analysis of the down-regulation of the hypoxic response upon re-oxygenation**

After re-oxygenation, RAP2.12:GFP protein was observed to disappear from the nucleus and the expression of low-oxygen genes decreased to normoxic levels (Licaisi et al. 2011b). In order to investigate the time required to degrade nuclear RAP2.12, plants expressing RAP2.12:GFP were first treated for 4 h with 1% oxygen and subsequently exposed to a normoxic atmosphere. At regular time intervals during 8 h of re-oxygenation, the nuclear localization of the fluorescent signal and the expression levels of the hypoxia responsive genes were measured. Between 3 and 4 h of re-oxygenation, RAP2.12:GFP disappeared from the nucleus (Fig. 4a,b). Gene expression analysis showed that already after 15 min of re-oxygenation, the expression of the hypoxia responsive genes declined and progressively reached normoxic levels after 4 h of re-oxygenation.

**DISCUSSION**

The modulation and timely control of plant responses to oxygen deficiency emerged as a crucial factor in the determination of plant susceptibility or tolerance. The mechanism of hypoxia perception in plants appeared to be fast in both the activation and termination of the anaerobic response. This is compatible with the post-transcriptional regulation of the oxygen sensing mechanism in plants, which mainly relies on the release of the RAP2.12 transcription factor protein from the plasma membrane concomitant with its stabilization and relocalization in the nucleus when the oxygen concentration is reduced. Two independent studies reported previously that 30–40 min of hypoxia or anoxia is sufficient to observe the induction of anaerobic genes (Loreti et al. 2005; van Dongen et al. 2009). Here, we confirmed this observation by qRT-PCR expression analysis of a selection of hypoxia response genes (Fig. 1c). However, the nuclear localization of RAP2.12:GFP chimeric protein was only observed after 3 h hypoxia (1% oxygen v/v; Fig. 1a,b). The Arabidopsis genome contains four additional ERF-VII transcription factors, including RAP2.2, a very close homolog of RAP2.12 (68.38% identity). Previous analyses employing transient trans-activation assays in protoplasts showed that RAP2.2 and RAP2.12 could induce anaerobic promoters several fold, whereas the effect of RAP2.3, HRE1 or HRE2 was much more modest or negligible (Licaisi et al. 2010, 2011c; Weits et al. 2014). The high similarity of the RAP2.2 and RAP2.12 in terms of amino acid, transcript abundance profile and gene trans-activation properties suggests that the two genes are functionally redundant and may be used to extend the information gath-
Figure 2. Localization of RAP2.12:GFP and hypoxia responsive gene expression upon different oxygen concentrations. (a) Subcellular localization in leaf epidermal cells of stably transformed GFP-fused RAP2.12 plants treated with different oxygen concentrations for 4 h. Treatments were performed in the dark (micrographs represent four biological replications). (b) Histogram showing the percentage of cells with nuclear localization of RAP2.12:GFP ($n = 3$). Each micrograph contained 20 cells on average. (c) Differential expression of hypoxia responsive genes after 4 h of hypoxic treatment. The oxygen concentration of each hypoxic atmosphere used is indicated on the x-axis. Data are mean values relative to reference (21% of oxygen = air) ± standard deviation ($n = 5$). Values marked with different letters are considered to be significantly different according to a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparison procedure ($P < 0.05$).

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The time discrepancy between visible accumulation of the GFP signal and the induction of the hypoxia response genes shown in Fig. 1 is likely explained by the low concentration of RAP2.12 transcription factor that is needed to induce the expression of these genes, whereas the intensity of the GFP signal can only be detected at much higher protein concentrations. Alternatively, the kinetics of RAP2.2 stabilization and nuclear localization is more rapid than that of RAP2.12.

A discrepancy between the expression of hypoxia response genes and RAP2.12 levels in the nucleus was observed during the re-oxygenation phase. Nuclear RAP2.12:GFP required 3 h of re-oxygenation to disappear from the nucleus, indicating the relocalization of photoconverted protein into the nucleus (indicated by white arrows). The micrographs are representative for at least three independent observations of three biological replicates. Scale bar, 10 μm.

(b) Green and red intensity measurements of RAP2.12:mEos fluorescence in the membrane and nucleus before photoconversion, after photoconversion and after hypoxia. Green bars represent the mean signal intensity of green fluorescence while red bars represent the red fluorescence intensity. Measurements were repeated three times on biologically independent replicate samples. The numbers above the bars represent the signal intensity ratio (red:green).

Figure 3. Translocation analysis of photoconverted RAP2.12:mEos protein. (a) Translocation of RAP:mEos protein upon hypoxia was analysed by photoconverting mEos from its green to red fluorescent form prior to the hypoxic (1% v/v O₂) treatment. A red fluorescent signal was observed in nuclei during hypoxia, indicating the relocalization of photoconverted protein into the nucleus (indicated by white arrows). The micrographs are representative for at least three independent observations of three biological replicates. Scale bar, 10 μm.
Figure 4. Subcellular localization of RAP2.12-GFP and gene expression analysis in stably transformed plants of *Arabidopsis thaliana* upon re-oxygenation. (a) Micrographs represent RAP2.12-GFP protein localization in normoxia (N), hypoxia (1% oxygen in the dark, 4 h) and re-oxygenation (1, 2, 3, 4 and 8 h) (n = 4). Scale bar, 10 μm. (b) Histogram represents the percentage of epidermal cells with nuclear localization of RAP2.12 (n = 3). Each micrograph contained 20 cells on average. (c) qRT-PCR analysis of gene expression level in plants treated with hypoxia [1%, (v/v) oxygen in air] and followed up to 8 h of re-oxygenation. Data are mean values relative to the reference treatment (normoxia) ± standard deviation. [Values marked with different letters are considered to be significantly different according to a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparison procedure (n = 5; P < 0.05)].
transcription factor RAP2.12 upon re-oxygenation, inhibition of transcription can also be achieved by a transcriptional repressor with similar DNA element affinity as the activators or able to bind regions in their proximity. Alternatively, it is possible that the ERF-VII activators are turned into repressors via post-translational modification or protein-protein interaction. Indeed, a trihelix transcription factor induced by RAP2.12 was recently shown to interact with it to repress the anaerobic response in a feedback-loop manner (Giuntoli et al. 2014). Because most of the genes encoding for these proteins are targets of RAP2.12, this would generate a feedback loop which is not unusual in the modulation of biological responses. A rapid switch-off of the hypoxic response is very likely to be crucial for plant tolerance, as constitutive anaerobic metabolism might reduce plant fitness in aerobic conditions. Moreover, the plant has to cope with a novel stress condition because sudden restoration of normoxia generates reactive oxygen species (Blokhina et al. 2003; Fukao et al. 2011).

Not only a fast response time is essential for plant tolerance to low oxygen, but also the sensitivity to react to changes in oxygen concentration is important. To assess the sensitivity of the molecular mechanism of oxygen perception, we characterized the localization of RAP2.12:GFP reporter construct after the exposure to different oxygen concentrations. The RAP2.12:GFP signal first appeared in the nucleus of cells that were incubated at 10% oxygen (Fig. 2a). In parallel, some hypoxia responsive genes (LBD41, PCO1) were also induced at 10% oxygen, while the others were induced at oxygen concentrations between 10 and 5%. This is consistent with microarray data in which moderate changes in transcript abundance were observed at 8% O2 (van Dongen et al. 2009). These results suggest that either various promoters possess different intrinsic responsiveness to RAP2.12 or that an additional transcriptional activator mediated the induction of the first set of genes at mild oxygen conditions. Interestingly, the two genes that exhibited early activation are possibly involved in the repression of the anaerobic response: the transcription factor LBD41 possesses an EAR domain that is described to be responsible for transcriptional repression (Ohta et al. 2001), while PCO1 and PCO2 are involved in the generation of the N-degron in the ERF-VII proteins (Weits et al. 2014).

Relocalization of RAP2.12 from the plasma membrane – where it is sequestered to during normoxic conditions via association with the membrane protein ACBP – was mainly suggested because in the original studies published by Licausi et al. (2011b), the fluorescent signal of RAP2.12:GFP disappeared entirely from the plasma membrane during hypoxia while the fusion protein accumulated in the nucleus. In the present study, we again observed nuclear accumulation of RAP2.12 when the oxygen availability decreased, but although the transgenic lines used were direct descendants of those reported in Licausi et al. (2011b), some membrane-associated signal remained even during a prolonged low oxygen treatment (Figs 1 & 2). The difference between the two experiments could be explained by our continuous selection for plants with strongest expression – and thus fluorescence – of the RAP2.12:GFP. Alternatively, the present data could also result from de novo RAP2.12:GFP synthesis during the hypoxic treatment. Therefore, we performed a photoconversion experiment with mEos-tagged RAP2.12. Prior to the hypoxic treatment, mEos was converted from its green to red fluorescent form. Subsequent appearance of red fluorescence in the nucleus (Fig. 3a,b) indicates that pre-existing RAP2.12 protein, within the cells prior to exposure to low oxygen, is imported into the nucleus upon hypoxia. However, apart from red fluorescence, green fluorescence could be observed in the nucleus during the low-oxygen stress (Fig. 3a,b). This can partly be explained by the incomplete photoconversion of mEos, but de novo RAP2.12 synthesis and subsequent nuclear translocation during hypoxia could also be responsible for appearance of RAP2.12:GFP in the nucleus. Taken together, this photoconversion experiment with RAP2.12:mEos confirms the earlier conclusion that the transcription factor RAP2.12 is activated upon hypoxia by relocalization from the plasma membrane to the nucleus. Future experiments using improved photoswitchable reporters will help in understanding the contribution of de novo synthesis of RAP2.12 in the activation of the anaerobic response. The use of protein synthesis inhibitors in combination with UV-induced photoconversion and hypoxic treatments in our hands resulted in extensive cell death and structure collapse, precluding de facto to draw conclusions from these experiments (data not shown).

With this study, we further characterized the role of RAP2.12 relocalization as part of the low-oxygen sensing mechanism in plants, as described previously by Licausi et al. (2011b) and Gibbs et al. (2011). Accumulation of RAP2.12 in the nucleus coincided with the induction of the hypoxia responsive marker genes and became apparent between 10 and 5% of oxygen in air. The reaction time of visible RAP2.12:GFP localization was much slower (4 h) than the induction of the molecular response, which was explained by the very low transcription factor concentration in the nucleus that is required to induce significant gene expression. Upon re-oxygenation, the decay of RAP2.12:GFP in the nucleus occurred simultaneous with the down-regulation of hypoxia responsive gene expression. Photoconversion of RAP2.12:mEos from green to red fluorescence prior to the hypoxic treatment provided strong evidence that relocalization of existing RAP2.12 from the plasma membrane into the nucleus occurs. However, de novo synthesis of the transcription factor cannot be excluded to happen during hypoxia as well.

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REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Comparison of RAP2.12::GFP fluorescence and chlorophyll autofluorescence.

Figure S2. Localization of RAP2.12::mEos in hypoxic conditions.

Figure S3. Nuclear localization of RAP2.12::mEos under hypoxia.

Figure S4. Effect of photoconversion of RAP2.12::mEos on its localization under aerobic conditions.

Figure S5. Effect of UV radiation treatment as used for plant transformation as used for plant transformation as used for plant transformation.

Table S1. List of primers used in this work (fw, forward primer; rv, reverse primer).