Ethylene plays an essential role in the recovery of Arabidopsis during post-anoaerobiosis reoxygenation

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ABSTRACT

Ethylene is known to play an essential role in mediating hypoxic responses in plants. Here, we show that in addition to regulating hypoxic responses, ethylene also regulates cellular responses in the reoxygenation stage after anoxic treatment in Arabidopsis. We found that expression of several ethylene biosynthetic genes and ethylene-responsive factors, including ERF1 and ERF2, was induced during reoxygenation. Compared with the wild type, two ethylene-insensitive mutants (ein2-5 and ein3eil1) were more sensitive to reoxygenation and displayed damaged phenotypes during reoxygenation. To characterize the role of ethylene, we applied microarray analysis to Col-0, ein2-5 and ein3eil1 under reoxygenation conditions. Our results showed that gene transcripts involved in reactive oxygen species (ROS) detoxification, dehydration response and metabolic processes were regulated during reoxygenation. Moreover, ethylene signalling may participate in regulating these responses and maintaining the homeostasis of different phytohormones. Our work presents evidence that ethylene has distinct functions in recovery after anoxia and provides insight into the reoxygenation signalling network.

Key-words: anoxia; ein2-5 and ein3eil1; re-aeration.

INTRODUCTION

Flooding or waterlogging leading to severe crop loss is a prominent agricultural issue in the face of climate change and recent worldwide abnormal weather patterns (Bailey-Serres et al. 2012b; Wheeler & von Braun 2013). Flooding in soil often exposes plants to hypoxic or anoxic stress, which results in a dramatic decrease in cellular energy, cytosolic acidification and generation of toxins from anaerobiosis. To withstand such unfavourable environments, plants have evolved sophisticated adaptive mechanisms to reinforce their hypoxic/anoxic tolerance (for reviews, see Bailey-Serres & Voesenek 2008; Bailey-Serres et al. 2012a,b). Gene expression profiling studies have revealed genes that are differentially regulated and uncovered signalling pathways triggered by hypoxia/anoxia (Klok 2002; Liu et al. 2005; van Dongen et al. 2009; Kreuzwieser et al. 2009; Hsu et al. 2011; Licausi 2011).

Ethylene plays an important role in the mediation of signalling events and participates in aerenchyma formation, adventitious root initiation, petiole/stem elongation and hyponasty response during flooding/waterlogging stress (Jackson 2008; Voesenek & Sasidharan 2013). It is also required, but not sufficient, to induce the expression of hypoxia marker gene ALCOHOL DEHYDROGENASE (ADH1) (Peng et al. 2001). Moreover, HYPOXIA RESPONSIVE ERFS1 (HRE1) and RELATED TO AP2.2 (RAP2.2), two essential transcription factors in the hypoxic signalling network of Arabidopsis (Columbia-0), are under the control of ethylene (Hinz et al. 2010; Yang et al. 2011). In rice (Oryza sativa), transcripts of the ethylene response factor (ERF)-like gene SUBMERGENCE1-A-1 (SUB1A-1) were increased in the presence of ethylene in a SUB1A-1-containing cultivar (Xu et al. 2006). Interestingly, two ERF-like genes, SNOKE1 (SKI) and SNORKE1 (SK2), are also regulated by ethylene in deepwater rice (Hattori et al. 2009). These ERFs are essential for the survival of rice under flooding/waterlogging stress.

After flooding subsides, the return to aerobic conditions after submergence under water poses a further challenge to plants. The sudden re-exposure to air may cause oxidative damage and impact plant cells (Pavelic et al. 2000; Rawyler et al. 2002; Blokhina et al. 2003). In animals, this post-anoxia/hypoxia recovery stage, designated as reoxygenation or re-aeration, has been well studied, as it commonly occurs along with many clinical symptoms, such as stroke and myocardial infarction (Kutala et al. 2007; Khan et al. 2010; Pundik et al. 2012). Massive reactive oxygen species (ROS) production is known to be a typical response when animal cells are re-exposed to air (Gauduel & Duvelleroy 1984; Zulueta et al. 1997). In anoxia-intolerant Iris germanica, a greater increase of the lipid peroxidation product, malondialdehyde (MDA), was detected at the recovery stage, implying that lipid peroxidation is a critical event during reoxygenation (Hunter et al. 1983). Later, many studies found that cellular damage during reoxygenation was mainly caused by the production of ROS (Albrecht & Wiedenroth 1994; Crawford et al. 1994; Pfister-Sieber & Brändle 1994; Blokhina et al. 1999). The activation of antioxidant systems to eliminate ROS has been observed during reoxygenation (Monk et al. 1987; Biemelt et al. 1998; Skutnik & Rychter 2009).

In rice, leaf cells become dehydrated within several hours of reoxygenation (Fukao et al. 2011). This suggests that lipid
peroxidation may cause membrane leakage, which renders plant cells unable to maintain their water status, leading to dehydration. Interestingly, in SUB1A-1-containing rice cultivars, SUB1A-1 could enhance the dehydration tolerance by inducing several abscisic acid (ABA)-responsive genes during the reoxygenation stage (Fukao et al. 2011). In addition to countering the effects of ROS, plants also have to re-adjust the physiological status from anaerobic to aerobic conditions. Metabolic and genetic analyses have shown that cellular events that occur during hypoxia, including anaerobic fermentation, a decrease in ATP/ADP ratio and selective mRNA translation, begin to return to normal status when plants are re-exposed to air (Branco-Price et al. 2008). Additionally, alanine accumulated in hypoxic tissues was degraded by activating alanine aminotransferase once plants escaped from anaerobiosis (Miyashita et al. 2007). Although many adaptive responses have been characterized in plants during reoxygenation, the signalling pathways leading to these responses remain largely unknown. Several studies have shown that mitogen-activated protein (MAP) kinase signalling is involved in reoxygenation in Arabidopsis (Chang et al. 2012) and that ABA-responsive genes are involved in mediating the dehydration response caused by reoxygenation in rice (Fukao et al. 2011).

It has been shown that the production of ethylene is increased in de-submerged or post-hypoxic Rumex (Voesenek et al. 1997, 2003). Recently, it was reported that ethylene was synthesized during reoxygenation in partially submerged cucumber roots (Garcia et al. 2013). However, the role of ethylene during reoxygenation is not fully understood. In the present study, we investigated the role of ethylene during reoxygenation. We used two ethylene-insensitive mutants, ein2-5 and ein3eill, to study the ethylene signalling during reoxygenation in Arabidopsis. Our data indicate that ethylene is necessary for the survival of Arabidopsis during reoxygenation. We used microarray assays to build up gene expression profiles of wild-type and ethylene-insensitive mutants during reoxygenation. Reoxygenation induces genes not only for responses to oxidative stress but also for dehydration response, ion transport and tricarboxylic acid (TCA) cycle replenishment. Transcriptomic comparison revealed that ethylene signalling might affect several significant responses and play roles in maintaining the balance of phytohormones during reoxygenation.

**MATERIAL AND METHODS**

**Plant materials, growth conditions and anoxia-reoxygenation treatment**

*Arabidopsis thaliana* accession Columbia-0 was used in this study. Ethylene-insensitive mutants ein2-5 and ein3eill were also used for phenotype observation and microarray assays. The ein2-5 is a null mutant of *ETHYLENE INSENSITIVITY2* (*EIN2*), which plays an essential role in ethylene signalling. It has a 7 base pair deletion in the coding sequence 939–945, which causes a frameshift mutation (Alonso et al. 1999). The ein3eill is a double mutant that is composed of two mutants: *ein3-1* (a G-to-A substitution at nucleotide 1598) and *eill-1* (an En-1 transposon insertion at nucleotide 697). Both mutants encode non-functional *ETHYLENE INSENSITIVE3* (*EIN3*) and *EIN3-LIKE1* (*EIL1*), leading to ethylene-insensitive phenotype (Alonso 2003). These mutant seeds were obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA, and have been confirmed through PCR-based genotyping (An et al. 2010). Seeds were sterilized by 0.5% sodium hypochlorite for 20 min and then sown on plates containing half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie BV, Haarlem, the Netherlands) with 0.55% phytagel and 0.5% sucrose. The plates were further stored at 4 °C in the dark for 3 d. After cold stratification, the plates were incubated at 22 °C and placed in a vertical position in a growth chamber under a 16 h light/8 h darkness photoperiod for 5 d with fluorescent lighting levels of 90 μE. Five-day-old seedlings were then transplanted onto fresh plates for another 2 d before anoxia treatment.

For anoxic treatment, 7-day-old Arabidopsis seedlings were placed into a gas chamber that was filled with pure nitrogen (100% N2) to replace the air in the chamber. To prevent remnant oxygen in the system, anaerobic bags (MGC AnaeroPack, Mitsubishi Gas Chemical Company, Tokyo, Japan) were placed in the chamber to ensure an anoxic environment. Firstly, the duration of anoxia that causes severe damage and complete lethality of Arabidopsis was determined. We found that anoxia for 10 h caused severe damage and most seedlings did not survive in 12 h of anoxic treatment. We therefore chose 4 and 8 h anoxic pretreatment durations for investigation of reoxygenation responses.

We initiated our 8 h anoxic treatment at the early stage of the light period (about 0800 h, while the sunrise was approximately 0600 h). To bring the treatment of 4 h anoxia-reoxygenation under the same circadian scheme, we initiated 4 h anoxic treatment at the middle stage of the light period (about 1200 h). The process of anoxia and reoxygenation was carried out in the dark. For re-aeration, air was pumped into the chamber to replace the nitrogen atmosphere.

**Phenotype observation and Evan’s blue viability test**

Seven-day-old Arabidopsis seedlings were placed in anoxic conditions for 4 h in a gas chamber and subjected to reoxygenation by refilling the chamber with air. For phenotype observation, chlorotic leaves were counted as an index of reoxygenation damage. For Evans blue staining, the shoot tissues at each reoxygenation time point were collected, weighed (fresh weight), and incubated in 0.1% Evans blue solution with shaking at 30 r.p.m. for 30 min. The stained leaf tissues were washed with de-ionized water to remove excessive dye. The de-stained tissues were disrupted through a steel ball in 500 μL of 0.1% SDS followed by the addition of 500 μL of de-ionized water. The solutions were centrifuged at 15 700 g for 30 min. The OD 590 nm of the supernatant was further measured using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Powerwave...
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RNA extraction and quantitative real-time PCR

Total RNAs from treated samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Based upon the manufacturer’s protocol, a 1:2 ratio of sample:TRIzol reagent was used to reach higher extraction efficiency. Five micrograms of extracted total RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primer to generate 20 μL of cDNA stock. The cDNA stock was then diluted 50-fold. qRT-PCR was performed with 9 μL of diluted cDNA, 0.5 μm of each primer and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Real-Time PCR Machine (Applied Biosystems). Sequences of primers used for qRT-PCR are listed in the Supporting Information File S1. Four biological repeats were performed for each time point. We adopted comparative Ct method to measure the expression levels of each genes (Schmittgen & Livak 2008). To normalize the data, TUB3 (AT5G62700) was used as an internal control. The expression level of target genes at normoxia in Col-0 was set as a reference (fold change = 1) to calculate the relative expression fold change at other time points or in different mutants.

RESULTS AND DISCUSSION

Ethylene is required for Arabidopsis seedlings to recover from anoxia

Our prior studies showed that ethylene is involved in mediating hypoxic responses in Arabidopsis. To investigate whether ethylene also played roles in cellular responses during the recovery from anoxic stress, we measured the transcript levels of genes that encode ethylene biosynthetic enzymes by qPCR. We found that the expression levels of several aminocyclopropane-1-carboxylic acid (ACC) synthase genes (ACSs) and one ACC oxidase gene (ACO2) were enhanced during the post-anoxia reoxygenation stage (Fig. 1a,b). Different ACS genes displayed different time-dependent expression patterns during reoxygenation. ACS6, ACS7 and ACS11 were induced at the early stages, whereas ACS2, ACS4 and ACS8 were induced at the late stages of reoxygenation (Fig. 1a). Interestingly, ACO1 was up-regulated in anoxia, but down-regulated upon re-aeration (Fig. 1b). This result suggests that the increased transcript levels of ACO1 at the end of anoxia might enhance the rate of ethylene biosynthesis once plants have been re-supplied with oxygen. Prompt biosynthesis of ethylene after hypoxia has been observed in Rumex in previous studies (Voosenek et al. 1997, 2003). In contrast to ACO1, ACO2 was mainly induced during reoxygenation (Fig. 1b). Moreover, two ethylene-responsive transcription factor (ERF) genes, ERF1 and ERF2, were induced during reoxygenation (Fig. 1c). ERF1 is a marker of the ethylene signalling pathway (Solano et al. 1998; Berrocal-Lobo et al. 2002; Lorenzo et al. 2003).
In addition, a recent report indicated that ERF1 also plays a role in various abiotic stresses (Cheng et al. 2013). In a prior study that investigated the profiling of polysomal mRNA, it was found that the transcript levels of ACS6, ACS7, ERF1 and ERF2 were increased in both steady-state and polysomal mRNA fractions during reoxygenation (Branco-Price et al. 2008). These results are consistent with our observation that ethylene biosynthesis and key transcriptional factors that mediate ethylene signalling responses were induced during reoxygenation.

The induction of ERF genes indicated the activation of ethylene signalling. To further verify this event, the responses of ethylene-insensitive mutants, ein2-5 and ein3eil1, were tested during reoxygenation. Seven-day-old Arabidopsis seedlings of Col-0, ein2-5 and ein3eil1 were subjected to anoxia for 4 h and then reoxygenated. Seven-day-old Arabidopsis seedlings of Col-0, ein2-5 and ein3eil1 were subjected to anoxia for 4 h and then reoxygenated. We found that some cotyledons and true leaves of ein2-5 and ein3eil1 began to enter chlorosis at 24 h post-anoxia (Fig. 2a). In contrast, Col-0 exhibited limited chlorosis after reoxygenation for 24 h (Fig. 2a). We further quantified the data through counting the number of chlorotic leaves, and measured the cell viability by Evans blue staining. Our data showed that reoxygenation caused much more cell damage in ein2-5 and ein3eil1 mutants (Fig. 2b,c), suggesting that ethylene plays a role in response to sudden oxygen re-exposure.

**Gene expression profiling during anoxia-reoxygenation**

We used microarray analysis to determine gene expression profiles of Col-0 during anoxia and reoxygenation. To distinguish between genes that are regulated by reoxygenation signalling events per se and those genes regulated by the anoxia signal, we applied anoxic treatment to Arabidopsis seedlings for 4 and 8 h, followed by reoxygenation treatment. Intriguingly, we found that even when we subjected plants to anoxic treatment for two different lengths of time, over half of the transcripts modulated during reoxygenation were similar (Fig. 3a). Through comparing the transcriptomic data...
of these two treatments, the genes whose transcripts changed (fold change ≥ 2 or ≤ 0.5, P-value < 0.1) at any reoxygenation time points (compared to the end of anoxia) under both conditions were defined as ‘reoxygenation-regulated genes’ (6751 genes) (Fig. 3b). We further classified these genes into three classes based upon their expression patterns and whether they were (1) up-regulated or (2) down-regulated during reoxygenation, respectively (Fig. 3c, Supporting Information File S2). Genes that were not significantly changed (fold change <2 or >0.5) during anoxia, but differentially expressed during reoxygenation, were classified as class I (Fig. 3c, Supporting Information File S2). Genes that were already induced or suppressed during anoxia (fold change ≥ 2 or ≤ 0.5) and whose expression patterns continued to show the change during the post-anoxia stage were classified as class II and class III (Fig. 3c, Supporting Information File S2). They represented the genes that were regulated by both oxygen deprivation and oxygen re-supply, illustrating a dynamic regulation of signalling in response to exposure to oxygen.

**A comprehensive strategy for re-adjustment of cellular homeostasis during reoxygenation**

To decipher the classified genes, GO analysis was conducted using the online bioinformatics software agriGO (Du et al. 2010). We imported each subclass of genes separately into the analysis toolkit and selected the enriched GO terms to elucidate how plants respond during reoxygenation. The enriched GO terms for class Ia, class IIa and class Ib are illustrated in Fig. 4. In addition, qPCR was applied to verify the genes selected from significant GO terms (Fig. 5).

In class Ia and class IIa, the enriched GO terms were mostly related to ROS. These GO terms included ‘response to high light’, ‘response to H2O2’, ‘response to ROS’ and ‘response to high temperature’ (Fig. 4a,b, Supporting Information File S3). The enrichment of these GO terms suggests that these genes are induced in response to potential increases in ROS during reoxygenation. This observation is consistent with most previous related reports (Pavelic et al. 2000; Rawyler et al. 2002; Blokhina et al. 2003). Within these GO terms, genes encoding heat shock factors (HSFs), heat shock proteins (HSPs) and antioxidants are the major genes (Figs 4a,b & 5a). Their activation might serve as a protective mechanism to eliminate damaged proteins or detoxify harmful ROS generated during reoxygenation. Interestingly, it has been reported that *HEAT SHOCK FACTOR A2* (HSFA2) was highly induced in anoxia and conferred tolerance to anoxia in plants (Banti et al. 2010). However, our data showed that HSFA2 and most HSPs and HSFs were mainly induced during reoxygenation (Figs 4b & 5a). These results imply that these HSFs/HSPs mainly play roles at later stages of reoxygenation.

Overaccumulation of ROS may lead to membrane damage and cause dehydration (Fukao et al. 2011). GO analysis showed that several GO terms related to dehydration are in class Ia (Fig. 4a, Supporting Information File S3). Several dehydration marker genes, such as *RESPONSIVE TO DESSICATION 29A* (RD29A), *KIN1*, *KIN2* and *DEHYDRATION RESPONSIVE ELEMENT BINDING (DREB)/COLD-RESPONSIVE ELEMENT BINDING (CBF)* genes, are included in this class (Figs 4a & 5b). Some of them are activated through the ABA-independent pathway (Lata & Prasad 2011). Moreover, several genes that respond to the ABA signalling were also found, such as *ABA INSENSITIVE 5* (ABI5), *MYB DOMAIN PROTEIN 2* (MYB2) and *MYC2* (Fig. 4a). These results indicate that both ABA-dependent and ABA-independent dehydration-related genes are activated during reoxygenation.

Another important issue in reoxygenation is the possible re-adjustment of energy utilization, and the enriched GO terms reflected this possibility. Many hypoxia/anoxia-induced marker genes, including *ADH*, *HREI*, *PDC* and *SUS*, were suppressed once plants were returned to aerobic conditions (see class Ib, Supporting Information Fig. S1A).
and Supporting Information File S3). Transient or rapid turning off of fermentation implied that plants could immediately receive signals to switch between aerobic and anaerobic respiration. Even though plants were able to recover their energy status in a short time, GO analysis showed that biogenesis was mostly slowed down in plants during reoxygenation. Genes in the GO terms such as ‘DNA replication’, ‘cell cycle’ and ‘ribosome subunit biogenesis’ were all down-regulated during reoxygenation (see class Ib, Supporting Information File S3). Besides those related to energy and

**Figure 3.** Reoxygenation causes dramatic changes in gene expression. Microarray assay was applied to investigate the gene expression profiling during 4 and 8 h anoxia and reoxygenation. (a) Plots of the gene expression. Left: 4 h anoxia and reoxygenation. Right: 8 h anoxia and reoxygenation. The arrow indicates the time point at which anoxic plants were exposed to air. (b) A Venn diagram of differentially expressed genes at 4 and 8 h reoxygenation. A gene showing a ≥2- or ≤0.5-fold change (normalized to normoxia, compared with Re0) at any reoxygenation time point was defined as a differentially expressed gene (DEG) in this study. Each section of the Venn diagram represents a distinct classification: blue section, genes only affected during 4 h anoxia and reoxygenation (2290, 17.76%); yellow section, genes only affected during 8 h anoxia and reoxygenation (3851, 29.87%); green section, genes were affected by both treatments (6751, 52.37%). (c) The 6751 DEGs in both 4 and 8 h anoxia and reoxygenation were categorized into three distinct classes according to their gene expression patterns. Class I: gene expression change was twofold or less during anoxia, but was twofold or more during reoxygenation. Class II: gene expression was up-regulated by twofold or more during anoxia, and continued to show a changed expression pattern during reoxygenation. Class III: gene expression was down-regulated by twofold or more during anoxia and showed a constitutively changed expression pattern during reoxygenation. Each gene set could be further divided into two subgroups, up- or down-regulation (twofold change), during reoxygenation.
Figure 4. Notable gene ontology (GO) terms in each class. GO analysis was conducted using the agriGO analysis toolkit on the website (http://bioinfo.cau.edu.cn/agriGO/) to investigate the significant enriched GO terms, \( P < 0.1 \). (a) GO terms represented in class Ia such as the ‘dehydration’ cluster that consists of the GO terms ‘response to salt’, ‘response to cold’, ‘response to drought’ and ‘ABA signalling’. The ‘ROS-I’ cluster includes the GO terms ‘response to high light’, ‘response to hydrogen peroxide’ and ‘response to heat’. The ‘ion transport’ cluster includes the GO terms ‘calcium transport’, ‘potassium transport’ and ‘cation transport’. The ‘JA’ and ‘ethylene’ cluster includes the GO terms for their biosynthesis and signalling transductions. (b) The GO terms represented in class IIa include the ‘ROS-II’ cluster consisting of the GO terms ‘response to high light’, ‘response to hydrogen peroxide’ and ‘response to heat’. The ‘ion transport’ cluster includes the GO terms ‘calcium transport’, ‘potassium transport’ and ‘cation transport’. The ‘JA’ and ‘ethylene’ cluster includes the GO terms for their biosynthesis and signalling transductions. (c) The GO terms represented in class IIIa include the ‘auxin’ cluster consisting of the GO terms of ‘response to auxin’ and ‘amino acid transport’. Ethylene signalling is essential in recovery from anaerobiosis.
Figure 5. Expression of selected genes from several notable gene ontology terms were validated by real-time PCR. Seven-day-old Arabidopsis seedlings that were treated with 4 h anoxia and then reoxygenated by air for 0, 0.5, 1, 3, 6 and 12 h (Re0, Re0.5, Re1, Re3, Re6 and Re12, Nor; normoxia) were used to measure the transcript levels by real-time PCR. Genes represented in each notable GO term were selected to validate the terms (a) reactive oxygen species (ROS) stimulus, (b) dehydration response, (c) ion transportation, (d) cellular metabolism and (e) mitochondria homeostasis. The data represent means ± SD from four biological repeats.
biogenesis, several GO terms closely related to ion transport were enriched in class Ia (Fig. 4a, Supporting Information File S3). The maker genes represented in the ion transport pathways that are differentially regulated during anoxia and reoxygenation. We found that the transcript level of PPCK1 catalysed the phosphorylation of PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC). PEPC is a key enzyme for photosynthesis in C₃ plants, which catalyzes the β-carboxylation of PEP to yield OAA and Pi. In non-photosynthetic tissues or C₄ plants, PEPC might play roles in pH homeostasis maintenance and replenishment of TCA cycle intermediates (Doubnerova & Ryslava 2011). We further found that PYRUVATE PHOSPHATE DIKINASE (PPDK) was also induced in re-aeration (Supporting Information Fig. S2A). PPCK1 catalyses the phosphorylation of PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE 1 (PPCK1) was suppressed in anoxia, but was immediately induced upon reoxygenation (Fig. 5d, Supporting Information S2A).

Replenishment of TCA cycle intermediates and mitochondria homeostasis maintenance during reoxygenation

We next checked for genes in primary metabolic pathways that are differentially regulated during anoxia and reoxygenation. We found that the transcript level of PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE 1 (PPCK1) was suppressed in anoxia, but was immediately induced upon reoxygenation (Fig. 5d, Supporting Information S2A). PPCK1 catalyses the phosphorylation of PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC). PEPC is a key enzyme for photosynthesis in C₃ plants, which catalyzes the β-carboxylation of PEP to yield OAA and Pi. In non-photosynthetic tissues or C₄ plants, PEPC might play roles in pH homeostasis maintenance and replenishment of TCA cycle intermediates (Doubnerova & Ryslava 2011). We further found that PYRUVATE PHOSPHATE DIKINASE (PPDK) was also induced in re-aeration (Supporting Information Fig. S2A). PPDK could catalyse the regeneration of PEP from pyruvate (Doubnerova & Ryslava 2011). The induc-
entry point into the TCA cycle is the conversion between α-ketoglutarate and glutamate, which can be catalysed in two different pathways. One is glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle, and the other is the NADH glutamate dehydrogenase (NADH-GDH) pathway (Miyashita & Good 2008). Interestingly, we also found that GDH1 and GDH2 were up-regulated during anoxia, but were induced to much higher levels during reoxygenation (Fig. 5d, Supporting Information Fig. S2B). In addition, GDH3 was up-regulated during reoxygenation, but was not induced during anoxia (Fig. 5d). The GDHs can catalyse the deamination of glutamate and are considered as key enzymes for nitrogen assimilation. However, recent data suggested that the main function of NADH-GDH is to provide α-ketoglutarate for the TCA cycle (Fontaine et al. 2012).

Up-regulation of GDHs during reoxygenation also implied that amino acid catabolism was enhanced to replenish the carbon source. We further checked the expression of genes for amino acid catabolic enzymes. As expected, the induction of ALANINE AMINOTRANSFERASE 2 (AlaAT2), BRANCHED-CHAIN AMINO ACID TRANSMINASE 2 (BCAT2), ISOVALERYL-COA-DEHYDROGENASE (IVDH), ALANINE:GLYOXYLATE AMINOTRANSFERASE 3 (AGT3), METHIONINE GAMMA-LYASE (MGL) and THREONINE ALDOLASE 1 (THAI) could be found in the array data (Supporting Information Fig. S2C). Taken together, these results suggest that plants need to replenish the carbon source loss from anoxia to ensure the operation of the TCA cycle (Supporting Information Fig. S2).

The other notable genes we found were the DICARBOXYLATE CARRIER (DIC) genes. The transcript of DIC2 was induced within 30 min of reoxygenation, and DIC1 and DIC3 were also induced at later stages (Fig. 5e). DIC proteins belong to the mitochondrial carrier protein family, which can transport a wide range of dicarboxylates, such as malate, OAA and succinate as well as phosphate, sulphate and thiosulphate (Palmieri et al. 2008). In animal systems, DICs can transport glutathione to detoxify ROS in mitochondria (Chen & Lash 1998). The early induction of DICs during reoxygenation suggests that plants promptly restore physiological homeostasis of mitochondria upon oxygen resupply.

**Ethylene modulates distinct pathways during reoxygenation**

To characterize ethylene-dependent signalling, we compared the gene expression profiles of Col-0, ein2-5 and ein3eil during reoxygenation. Because we used a single-colour expression chip (see the Materials and Methods section), we normalized all the genes from Col-0, ein2-5 and ein3eil to the normal growth conditions (Nor) of Col-0 to find the comparative fold changes for each gene. Then we compared the fold change between Col-0 and the two mutants at every reoxygenation time point. If there was a twofold or greater change (P-value < 0.1) in the mutant gene expression in comparison with Col-0 at any reoxygenation time point, we defined the gene as a differentially expressed gene (DEG). Following this method of analysis, we obtained ein2-5-dependent DEG and ein3eil-dependent DEG lists. As both ein2-5 and ein3eil are ethylene-insensitive mutants, we further constructed a Venn diagram to show the overlapping part of the two DEG lists to study ethylene-dependent signalling (1782 genes) (Fig. 6a, Supporting Information File S4). Analysis of these DEGs showed that loss of ethylene signalling during reoxygenation caused a clear change in several GO terms that were characterized in the previous sections. According to the functional similarity, we further categorized these GO terms into five groups: ‘ethylene’, ‘JA’, ‘ABA’, ‘heat’ and ‘dehydration’ (Fig. 6b, Supporting Information File S5). We found that most genes in ein2-5 or ein3eil had higher expression levels than in Col-0 (Fig. 6b). To verify these gene expression patterns, we conducted qPCR to confirm several genes represented in each group (Figs 7 & 8).

In the ‘JA’ group, the representative marker genes are JA biosynthetic genes and IAZ genes, including LOX3, LOX4, AOC3, OPR3 and OPC1, as well as IAZ1, IAZ5, MYB15 and MYC2. All these genes showed higher expression levels in ein2-5 and ein3eil, suggesting that ethylene might inhibit JA function during reoxygenation (Fig. 7). Interestingly, a previous study showed the antagonism between ethylene and JA in ozone-induced cell death (Tuominen et al. 2004). Although JA signalling may negatively mediate PCD and positively regulate antioxidant system under stresses, JA signalling is also required for PCD (Reinbothe et al. 2009). Based upon the multi-functionality of JA, we suggest that ethylene might play roles in fine-tuning JA responses to react in proper manner during reoxygenation. In the ‘ethylene’ group, the representative markers are ACS7, ACS11, ERS1 and ERF2. In ein2-5 and ein3eil, ACS7 and ACS11, which are ethylene biosynthetic genes, had higher expression levels. However, ERS1 and ERF2 genes, which are involved in ethylene signalling, had lower expression levels in ein2-5 or ein3eil during reoxygenation. Ethylene seems to suppress its own biosynthesis by a feedback mechanism, but enhances its signal transduction during reoxygenation (Fig. 8a). In the ‘ABA’ group, the representative markers are NCED3, NCED5, NCED9, RD20, RD22, KIN1, KIN2 and TSPO, all of which showed higher expression levels in ein2-5 or ein3eil (Fig. 8b). Antagonism between ABA and ethylene was observed during hypoxia (Bailey-Serres et al. 2012b). Our results suggest that ethylene also negatively regulates ABA functions during reoxygenation.

In the ‘heat’ group, several HSFs and HSPs showed higher expression levels at 3 and 6 h of re-aeration in the array data (Fig. 7b). Interestingly, in qPCR validation, we found that HSFA2 had a lower expression level in ein2-5 and ein3eil in the first 30 min to 1 h of reoxygenation, but had a higher expression level at later stages of reoxygenation (3–6 h). Several other HSPs, such as Hsp17.6, Hsp21 and HSP70, also showed a similar pattern (Fig. 8c). These results suggest that ethylene might play a role in regulating the expression of these heat responsive genes during reoxygenation. In the ‘dehydration’ group, several dehydration-related genes such as MYB102, MYB121 and P5CS1 also showed higher expression...
levels in ethylene-insensitive mutants. However, most of these genes are also under ABA regulation under conditions of dehydration. This suggests that ethylene may suppress part of the dehydration response through the inhibition of ABA signalling.

Ethylene signalling is involved in various responses in reoxygenation

As GO analysis suggested that ethylene may inhibit ABA signalling during reoxygenation, we further characterized several DREB/CBF genes that were suppressed when ethylene signalling was absent. Characterized genes included DREB1A/CBF3, DREB1B/CBF1, DREB2A and DREB2B (Fig. 8d). These results suggest that although ethylene suppresses ABA signalling, these transcription factors, which are ABA-independent regulators, were activated by ethylene to deal with the dehydration caused by reoxygenation.

As a key phytohormone in reoxygenation, ethylene also influenced the expression of several marker genes. In the metabolic pathway, ethylene signalling is required for the
activation of PPDK, IVDH and THAI during reoxygenation (Fig. 8e). This suggests that ethylene may be involved in the replenishment of TCA cycle intermediates. FORMATE DEHYDROGENASE (FDH; EC 1.2.1.2.) is another gene affected in ethylene mutants (Fig. 8e). As an important mitochondrial enzyme, FDH catalyses the oxidation of formate into CO₂. It participates in the alternative pyruvate conversion during fermentation, which can cause less alcohol production during anaerobic growth in bacteria. However, it has not been proven that this alternative pathway exists in higher plants. On the contrary, formate can be generated through a non-enzymatic pathway – the oxidation of glyoxylate. Moreover, formate is also toxic to mitochondria (Liesivuori & Savolainen 1991). Since oxygen deprivation would activate genes in glyoxylate cycle, which may lead to the synthesis of glyoxylate in plants (Lu et al. 2005; Hsu et al. 2011), we propose that the oxidation of glyoxylate should happen during reoxygenation. Thus, the activation of FDH could further convert formate into CO₂ during reoxygenation. This implies that ethylene may play a critical role in the regulation of mitochondria homeostasis.

The expression of the gene STELAR K+ OUTWARD RECTIFIER (SKOR) was affected in ethylene mutants (Fig. 8e). SKOR is a potassium channel that mediates the delivery of potassium from stelar cells to the xylem. It has been suggested that the SKOR channel is closed when plants suffer hypoxia/anoxia (Colmer & Greenway 2011). Therefore, to balance the ion homeostasis in plant cells, the level of SKOR should be increased during reoxygenation. Taken together, these results suggest that ethylene signalling might also influence potassium ion homeostasis regulation.

CONCLUSION

Our transcriptomic analyses suggest that a complicated regulatory network is triggered in Arabidopsis during reoxygenation after anoxic treatment. We propose that ROS may play a dominant role in the initiation of various responses, such as mitochondria homeostasis maintenance, dehydration and heat repair response (Fig. 9). In addition, many aspects of metabolic processes were also fine-tuned during reoxygenation, including glycolysis, fermentation, TCA cycle replenishment, ion homeostasis and cellular biogenesis.

Our prior studies indicated that ethylene plays an essential role in regulating hypoxic responses (Peng et al. 2001; Hsu et al. 2011; Yang et al. 2011). The current study showed that ethylene also plays an essential role in reoxygenation after anoxic stress (Fig. 9). Our transcriptomic analyses of wild-type and ethylene-insensitive mutants suggested that ethylene mainly plays a role in maintaining the balance of hormone signalling, including ethylene, as well as ABA and JA. In addition, ethylene mediated the regulation of several different classes of genes, including DREBs, HSFs and HSPs, and the metabolic genes PPDK, THAI and IVDH, suggesting the involvement of ethylene in regulating metabolic pathways during reoxygenation.

Our data also showed that the regulation of genes involved in glycolysis, fermentation, the TCA cycle, ion homeostasis and cellular biogenesis during reoxygenation was independent of ethylene signalling. The transcript levels of several hypoxia marker genes, such as ADH and PDC, were decreased once plants were exposed to air, suggesting that energy utilization was shifted to aerobic respiration. Consistent with this idea, the expression of genes in the TCA cycle was induced during reoxygenation. PPCK1 and three GDH genes were activated during reoxygenation, suggesting that they might play important roles in TCA cycle intermediate replenishment. However, the transcript levels of genes related to biogenesis (i.e. DNA replication, ribosome biogenesis, cell cycle) were decreased, suggesting that even when plants started to generate energy more efficiently by replenishing TCA cycle intermediates, growth or cell proliferation might still be slow until the plants have completely recovered from damage sustained during anoxia.

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Figure 8. Expression of genes that were influenced in ein2-5 and ein3eill mutants. Seven-day-old Arabidopsis seedlings of Col-0 and mutants were subjected to anoxia for 4 h in a gas chamber and then reoxygenated in air for 0, 0.5, 1, 3, and 6 h (Re0, Re0.5, Re1, Re3 and Re6). The transcript levels at each time point including normoxia (Nor) were quantified by real-time PCR. (a) Ethylene biosynthesis genes (ACS7 and ACS11) and ethylene signalling markers (ERSI and ERF2). (b) ABA biosynthesis and signalling genes: three NCED genes (NCED3, 5 and 9) and some ABA-mediated genes (TPSO, RD20, RD22, KIN1 and KIN2). (c) HSFs and HSPs: HSF2, HSP17.6A, HSP21 and HSP70; (d) DREB genes: DREB1A and IB. (e) Genes involved in different physiological processes: FORMATE DEHYDROGENASE (FDH), PYRUVATE PHOSPHATE DIKINASE (PPDK), STELAR K+ OUTWARD RECTIFIER (SKOR), ISOVALERYL-COA-DEHYDROGENASE (IVDH) and THREONINE ALDOLASE 1 (THAI). The data represent means ± SD from four biological repeats. *P < 0.05 and **P < 0.01 in Student’s t-test.

Figure 9. Schematic representation of signalling pathways triggered during reoxygenation. Regulatory pathways were constructed based upon transcriptomic analyses and RT-PCR. Arrows indicate positive effects of the upstream and ↓ indicates negative effects.
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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. Notable gene ontology terms in each class – continued.

Figure S2. Replenishment of TCA cycle intermediates.

File S1. Sequences of primers for qRT-PCR.

File S2. Genes differentially expressed during anoxia-reoxygenation in Col-0.

File S3. Gene ontology analysis of the genes classified in each subclass in Col-0.

File S4. Genes differentially expressed in Col-0, ein2-5 and ein3eil1 during reoxygenation.

File S5. Gene ontology analysis of the genes differentially expressed in Col-0, ein2-5 and ein3eil1 during reoxygenation.
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