Cross-Kingdom Comparison of Transcriptomic Adjustments to Low-Oxygen Stress Highlights Conserved and Plant-Specific Responses[1][W][OA]

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High-throughput technology has facilitated genome-scale analyses of transcriptomic adjustments in response to environmental perturbations with an oxygen deprivation component, such as transient hypoxia or anoxia, root waterlogging, or complete submergence. We showed previously that Arabidopsis (*Arabidopsis thaliana*) seedlings elevate the levels of hundreds of transcripts, including a core group of 49 genes that are prioritized for translation across cell types of both shoots and roots. To recognize low-oxygen responses that are evolutionarily conserved versus species specific, we compared the transcriptomic reconfiguration in 21 organisms from four kingdoms (Plantae, Animalia, Fungi, and Bacteria). Sorting of organism proteomes into clusters of putative orthologs identified broadly conserved responses associated with glycolysis, fermentation, alternative respiration, metabolite transport, reactive oxygen species amelioration, chaperone activity, and ribosome biogenesis. Differentially regulated genes involved in signaling and transcriptional regulation were poorly conserved across kingdoms. Strikingly, nearly half of the induced mRNAs of Arabidopsis seedlings encode proteins of unknown function, of which over 40% had up-regulated orthologs in poplar (*Populus trichocarpa*), rice (*Oryza sativa*), or *Chlamydomonas reinhardtii*. Sixteen *HYPOXIA-RESPONSIVE UNKNOWN PROTEIN* (*HUP*) genes, including four that are Arabidopsis specific, were ectopically overexpressed and evaluated for their effect on seedling tolerance to oxygen deprivation. This allowed the identification of *HUPs* coregulated with genes associated with anaerobic metabolism and other processes that significantly enhance or reduce stress survival when ectopically overexpressed. These findings illuminate both broadly conserved and plant-specific low-oxygen stress responses and confirm that plant-specific *HUPs* with limited phylogenetic distribution influence low-oxygen stress endurance.

Oxygen is required for the efficient production of ATP by plants and other aerobes. Despite the extremely high affinities for oxygen of the oxidases involved in aerobic respiration (K_m of 0.08–1 μM; Hoshi et al., 1993), obligate and facultative aerobes regularly experience oxygen deprivation for myriad reasons. Although oxygen is a by-product of photosynthesis, plants lack a circulatory system to mobilize oxygen to heterotrophic roots, tubers, meristems, germinating pollen, and developing seeds. These and flooded organs are vulnerable to oxygen deficiency. In mammals, intermittent tissue or cellular hypoxia can occur due to inhibition of pulmonary respiration (i.e. sleep apnea; Azad et al., 2009) and blood flow (i.e. stroke; Mense et al., 2006), a low-oxygen environment (i.e. high altitude), or high cellular density and metabolic activity (i.e. tumor cells [Chen et al., 2008] and ischemia [Fang et al., 2009]). In the case of microbes, oxygen availability is influenced by the density and identity of surrounding organisms and can be modulated over the course of a day or season, as observed in the microbial mats found in thermal springs (Steunou et al., 2006). It is not surprising that organisms can be predisposed to endure or avoid a low-oxygen environment. For example, submergence-tolerant rice (*Oryza sativa*) varieties adopt a quiescence strategy that limits the consumption of energy reserves and growth when covered by deep floodwaters, whereas deepwater rice varieties that experience a progressive deep flood during establishment can accelerate elongation growth to extend leaves above water (Fukao et al., 2006; Hattori et al., 2009). Other constitutive or

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induced adaptations of plants to oxygen deficiency include the formation of aerenchyma connecting aerial and underwater organs and alterations in organ or subcellular architecture of roots and leaves to oxygen status (Bailey-Serres and Voesenek, 2008). Animals also display adaptations that improve oxygenation, ranging from changes in vascular physiology and development (Bertout et al., 2008; Bishop-Bailey, 2009) to the formation of gills (van der Meer et al., 2005).

At the cellular level, a common eukaryotic response to oxygen deficiency is the Pasteur effect, whereby glycolysis and fermentation are promoted and the tricarboxylic acid (TCA) cycle and mitochondrial respiration are repressed (animals [Hochachka et al., 1996; Papandreou et al., 2006], yeast [Kwast et al., 2002], and plants [Bailey-Serres and Voesenek, 2008]). However, some organisms greatly curtail energy consumption to conserve precious reserves and extend survival during prolonged oxygen deficiency (i.e. turtles [Bickler and Buck 2007] and rice [Bailey-Serres and Voesenek, 2008]). Low-oxygen-induced metabolic adjustments involve sensing and signaling mechanisms that precipitate complex reconfiguration in gene expression that are far better understood in animals, fungi, and bacteria than in plants (Bruick, 2003; Bailey-Serres and Chang, 2005). The production and detoxification of reactive oxygen species (ROS) is likely to play a widespread role in signaling and damage at the onset and release from low-oxygen stress (Bailey-Serres and Chang, 2005; Rhoads and Subbaiah, 2007; Semenza, 2007).

Genome-scale technologies facilitate the appraisal of responses to environmental stimuli in a wide variety of organisms but are generally used to decipher responses within a single species. The interrogation of multispecies data sets within and across kingdoms can recognize evolutionarily conserved and species-specific responses to environmental perturbation. A meta-analysis of transcriptomic data from five species exposed to hydrogen peroxide stress identified common transcriptional adjustments, including one protein family globally induced in prokaryotes and eukaryotes (Vandenbroucke et al., 2008). Despite the growing number of independent evaluations of plant (Arabidopsis [*Arabidopsis thaliana*], poplar [*Populus trichocarpa*], rice, and *Chlamydomonas reinhardtii*), animal, fungal, and bacterial responses to low oxygen, there has been no systematic comparison of responses across plant species, kingdoms, or experimental systems. Here, we took advantage of 34 published microarray data sets that record the responses to varying degrees of oxygen deficiency of 21 organisms, representing plants, animals, fungi, and bacteria. The coupling of gene expression data with protein ortholog assignments exposed conserved and species-specific responses. The regulation of mRNAs associated with core cellular responses, including reconfiguration of central carbon metabolism and induction of heat shock proteins (HSPs), was generally preserved across organisms. By contrast, signaling protein and transcription factor (TF) genes responding to the stress were largely plant or species specific. We also considered genes encoding hypoxia-responsive proteins of unknown function that were highly coexpressed with genes associated with the core metabolic adjustments but varied in species distribution (Horan et al., 2008). We confirmed that transgenic Arabidopsis seedlings that ectopically express *HYPOXIA-RESPONSIVE UNKNOWN PROTEIN* (*HUP*) genes exhibit altered endurance of low-oxygen stress.

RESULTS AND DISCUSSION

Data Collection and Identification of Differentially Expressed Genes

Our initial objective was to perform a meta-analysis of alterations in mRNA abundance in response to transient oxygen deprivation in evolutionarily distant species, including model monocot and eudicot, animal, fungal, and bacterial species. Publicly available gene expression data were obtained for 21 species, in which stress conditions varied from anoxia to partial oxygen deprivation (i.e. hypoxia, 0.1%–10% oxygen; Supplemental Table S1). The plant survey included experiments that monitored the responses of Arabidopsis seedlings to varying degrees of hypoxia, poplar roots to flooding, rice seeds and coleoptiles to anoxia, rice seedling leaves to submergence, and *Chlamydomonas* cells to anoxia. Gene transcripts that were significantly up- or down-regulated in response to the experimental treatments were identified as differentially expressed genes (DEGs) by uniform processing of the data sets, using 1.8- or 2-fold change in abundance and a false discovery rate of 0.1 to 0.01 as criteria for differential expression (Supplemental Tables S2 and S3). In cases where raw microarray data were unavailable, published confidence values were used.

The overall number of DEGs in each experiment and organism varied as expected for a data set comprising diverse experimental parameters and species (Fig. 1A; Supplemental Table S1). Notably, the highest numbers of DEGs prevailed in plant samples, with up to 2,996 induced and 3,042 reduced mRNAs. In most cases, the number of induced DEGs exceeded the number of reduced DEGs; however, the number of reduced DEGs was greater when treatment duration was extended. Most animal data sets identified fewer than 100 DEGs, with the exception of stagnant human cell cultures (more than 1,000 DEGs; Guimbellot et al., 2009). DEG numbers in microorganisms were similar to those of animals, despite their lower gene contents. Notably, the facultative anaerobes *Saccharomyces cerevisiae* and *Escherichia coli*, which reconfigure metabolism and sustain growth under low oxygen, displayed the largest number of DEGs of the microorganisms sampled. Two other trends were observed: DEG number increased with the severity of stress in plants and bacteria, and it was influenced by the duration of the

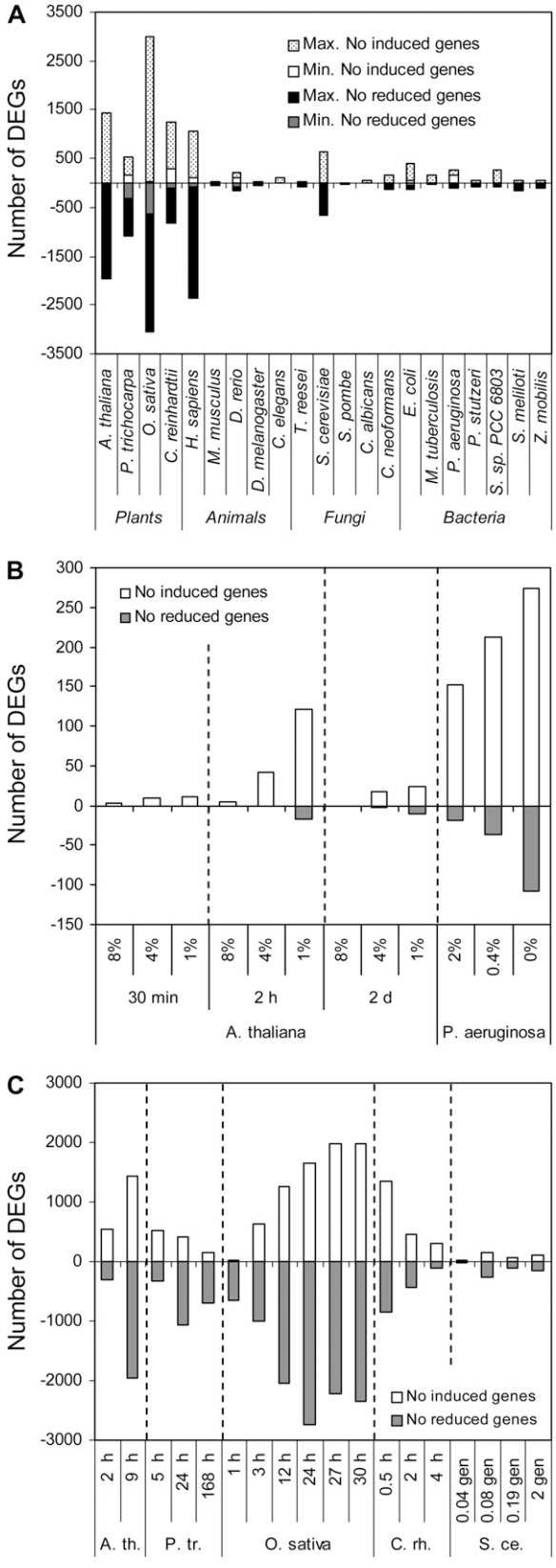

Figure 1. Number of induced and repressed genes following oxygen deprivation in diverse organisms. A, Bars indicate the minimum and maximum number of DEGs recognized when multiple studies were evaluated (Supplemental Table S1). Induced DEGs are plotted as positive numbers; reduced DEGs are plotted as negative numbers. Species investigated are as follows: *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Chlamydomonas reinhardtii*, *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Trichoderma reesei*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Cryptococcus neoformans*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Synechocystis* species PCC 6803, *Sinorhizobium meliloti*, and *Zymomonas mobilis*. B, Responses of organisms over a range of oxygen concentrations and time. C, Responses of organisms over time. gen, Generation.

treatment in plants and to a lesser degree in yeast (Fig. 1, B and C; Supplemental Table S1). This overview confirms that low-oxygen stress induces a programmed transcriptomic response in organisms of four kingdoms, with the most dramatic reconfiguration in plants. This marked adjustment of the plant transcriptome most likely reflects complex strategies of acclimation and adaptation.

Recognition of Orthologous Induced and Reduced Genes

To compare the transcriptomic response across these diverse species, it was necessary to identify genes encoding evolutionarily related proteins. To accomplish this, the proteomes of the 21 organisms were coclustered into protein families using the OrthoMCL method (OMCL), which performs an all-against-all BLAST comparison of protein sequences with subsequent Tribe-Markov clustering (Li et al., 2003). The analysis of 367,643 proteins in total resolved 42,830 clusters of related proteins with two to 2,185 proteins per OMCL cluster (Supplemental Tables S4 and S5). For simplicity, the proteins within a single cluster (family) are termed orthologous, although conservation in function was not tested. We found that clusters with only two or three proteins were the most frequent (33% and 21% of all clusters, respectively), with the frequency decreasing with the number of proteins per cluster (Fig. 2, A and B). Clusters with more than 40 proteins included highly expanded gene families in one or a few organisms, such as the transposable element proteins of rice and known evolutionarily conserved proteins including glycolytic enzymes (glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, enolase), ribosomal proteins, tRNA synthetases, RNA polymerases, and ATP-binding cassette transporters (Supplemental Table S4). Protein clusters with representatives from a few species were the most frequent, as recognized previously (Li et al., 2003; Vandenbroucke et al., 2008). For example, 40% of the clusters contained genes from one organism, whereas only 0.4% possessed entries from all 21 organisms (Fig. 2C). Over 1,000 clusters (2.4%) included entries from the 14 eukaryotes in the data set, and 38 clusters (0.1%) included exclusively entries from the seven prokaryotes evaluated. The analysis recognized 19,122 clusters

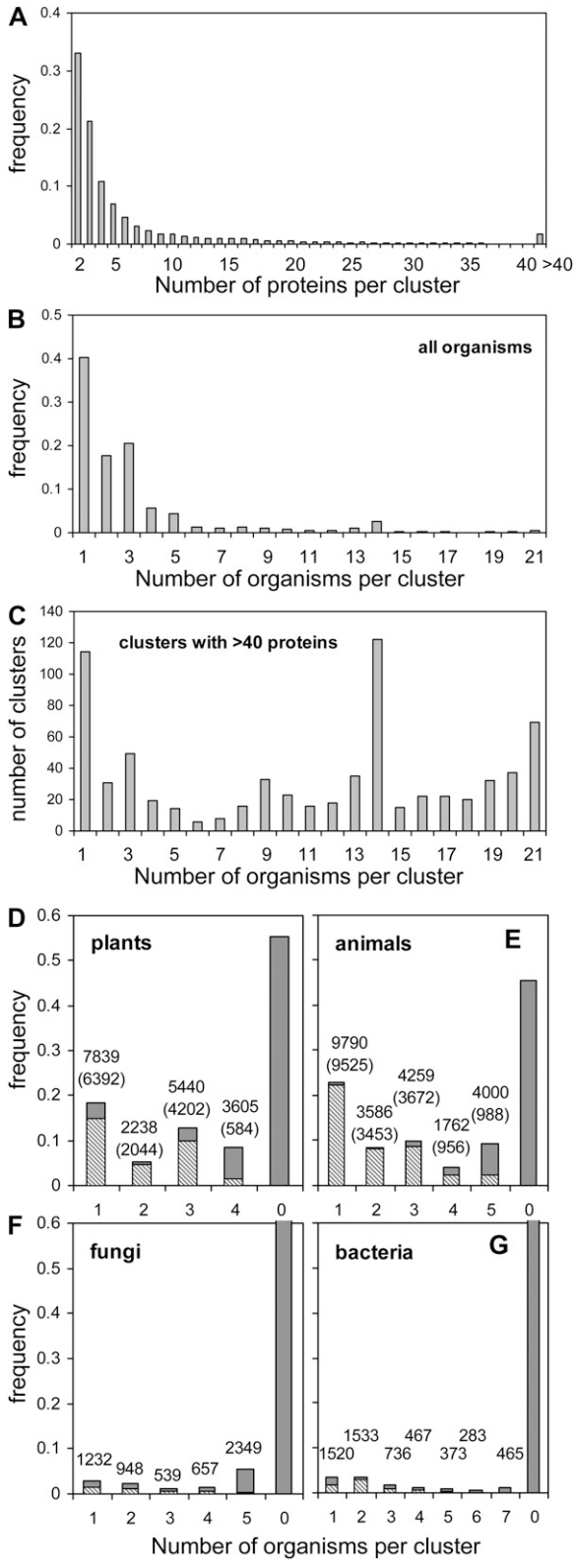

Figure 2. OMCL clustering of 367,643 proteins from 21 organisms resolved 42,830 clusters/protein families. A, Cluster size frequency. B, Frequency of number of organisms per cluster. C, Distribution of clusters with more than 40 genes across organisms. D to G, Frequency of organisms per cluster in individual kingdoms. The 0 bar indicates clusters not identified in the kingdom. Values above bars are numbers of protein clusters. Lighter gray regions indicate frequency of clusters limited to kingdom, with numerical values in parentheses.

with plant genes (Supplemental Table S4). Of these, only 8.4% (3,605) had entries in all four species (Fig. 2D), and most of these clusters (84%) also included proteins from nonplant species. By contrast, clusters limited to one to three higher plants were predominantly kingdom specific (Fig. 2D, lighter gray sections of bars). For example, Arabidopsis proteins were present in 11,806 clusters, from which 8,951 (75%) were conserved across higher plants and 1,066 (9%) were Arabidopsis specific. Similar trends were evident for animal clusters (Fig. 2E).

The small clusters of evolutionarily distinct proteins are likely to reflect the ongoing ontogeny and evolution of proteins. Indeed, proteins of unknown function (PUFs) and proteins of no defined motif tend to be species specific (Gollery et al., 2006; Horan et al., 2008). Reflective of this is the observation that 30% (3,595) of the low-oxygen-induced DEGs and 25% (3,353) of the reduced DEGs found in one of the four plants evaluated encode a PUF (Supplemental Fig. S1; Supplemental Table S6). Although species-specific stress-induced PUFs can play a role in abiotic stress tolerance (Luhua et al., 2008), the approximately 450 cross-species-conserved hypoxia-responsive unknown proteins (e.g. HUPs) that are induced in multiple plant species are attractive targets for reverse genetic analysis.

Broadly Conserved Induced and Reduced Genes

To recognize evolutionarily conserved versus species-specific responses to oxygen deprivation, 12,685 induced and 15,966 reduced DEGs were sorted to an OMCL cluster, and enrichment of specific clusters was tested by hypergeometric distribution analyses (Supplemental Table S6). This identified 2,409 clusters that contained two or more induced DEGs. Twenty percent (466 clusters) included members from three or more organisms. The cluster with induced DEGs from the greatest number of organisms (11 species, four kingdoms) encoded sugar transporters; enzymes of primary carbon metabolism, secondary metabolism (i.e. coproporphyrinogen III oxidase, C-5 sterol desaturase, squalene monooxygenase), HSPs, and enzymes that ameliorate ROS were also induced in the majority of the species (Supplemental Fig. S2; Supplemental Table S6b). Mitogen-activated protein kinases (MAPKs) were the only signal transduction pathway component induced in diverse eukaryotes (plants, human, and yeast). The broadly reduced DEGs resolved into 3,136 clusters with two or more proteins, of which 719 clusters contained sequences from three or more organisms (Supplemental Fig. S2; Supplemental Table S6d). The most frequently represented clusters encoded ribosomal proteins, TCA cycle enzymes, and aerobic respiratory complex subunits (i.e. isocitrate

dehydrogenase, F_0-ATPase δ-subunit; Supplemental Figs. S2 and S3). Gene Ontology (GO) analysis confirmed that many species reduced mRNAs encoding proteins associated with aerobic respiration (2.77E-12) and biogenesis of ribosomes (7.59E-34), organelles (1.95E-09), and cell walls (1.06E-11; Supplemental Table S7d). The decline in mRNAs associated with biosynthetic processes strongly suggests that restriction of ATP-consuming processes is an evolutionarily conserved coping mechanism. Interestingly, a reduction in mRNA abundance is not a prerequisite for down-regulation of gene expression, since the transient selective repression of translation occurs in both animals and plants during low-oxygen stress (Thomas and Johannes, 2007; Branco-Price et al., 2008).

Diversity in Anaerobic Energy Production

In general, oxygen deprivation reconfigures central carbon metabolism to promote substrate-level ATP production (Fig. 3; Supplemental Fig. S4). Frequently, glycolysis is activated and the excess NADH is recycled through fermentative metabolism of pyruvate, regenerating the NAD^+ needed to maintain glycolytic flux. However, there is remarkable diversity in anaerobic metabolism. In animals, anaerobic metabolism of pyruvate is limited to the formation of lactate (lactate dehydrogenase in OMCL2360; Supplemental Table S6). In plants, lactate is not the major fermentation end product; rather, pyruvate is predominantly converted to acetaldehyde by pyruvate decarboxylase (PDC; OMCL1074), which is further metabolized to ethanol by alcohol dehydrogenase (ADH; OMCL198 and OMCL286; Branco-Price et al., 2008; van Dongen et al., 2009). Consistently, *PDC* and *ADH* were induced in rice, Arabidopsis, and poplar. Plants also produce Ala (Ala aminotransferase [OMCL240] and Asp aminotransferase [OMCL347]) and γ-aminobutyric acid (Glu decarboxylase [OMCL595]) and accumulate succinate under low-oxygen stress (Bailey-Serres and Voesenek, 2008). *PDC* and *ADH* were also induced in the five fungal species evaluated, which produce ethanol under anaerobiosis (Fig. 3; Supplemental Table S6). In bacteria, pyruvate metabolism is considerably more complex. It can be converted into acetyl-CoA and formate by pyruvate-formate lyase (OMCL17942) or to acetyl-CoA and hydrogen by pyruvate-ferredoxin oxidoreductase and hydrogenases (OMCL12153, OMCL22614, and OMCL40976). Acetyl-CoA can be further metabolized into acetate by the sequential action of phosphate acetyltransferase (OMCL6584) and acetate kinase (OMCL4953) or into ethanol by ADH-E (OMCL1642). The algae *Chlamydomonas* presents a special case, in which anaerobic metabolism of pyruvate can be metabolized to lactate or ethanol via the pathways characteristic of higher plants as well as to formate, hydrogen, and acetate by the pathways characteristic of bacteria (Mus et al., 2007; Fig. 3).

Microorganisms can additionally produce nitrogen/nitric oxide to facilitate anaerobic survival (nitrate reductase [NR], OMCL10813, OMCL10728, and OMCL16045; nitrite reductase, OMCL34060; nitric oxide reductase, OMCL23279 and OMCL15967; nitrous oxide reductase, OMCL23273 [Sherman et al., 2001; Alvarez-Ortega and Harwood, 2007]). Interestingly, plants induce NRs under low-oxygen stress (OMCL130), although the enzyme was distinguished from the bacterial NRs by OMCL analysis. A function of plant NR could be, as for bacterial NRs, the removal of reduced NADH. Accordingly, plants survive low-oxygen stress for longer periods if nitrate is provided rather than ammonia (Fan et al., 1997; Allègre et al., 2004). NR mutants of tobacco (*Nicotiana tabacum*) are also more sensitive to hypoxia than the wild type (Stoimenova et al., 2003).

Substrate-level ATP production requires the catabolism of soluble or stored carbohydrates (i.e. Suc and starch, or glycogen in animals). To circumvent the exhaustion of soluble carbohydrate reserves, enzymes responsible for the cleavage of stored carbohydrates such as glycogen phosphorylases and related proteins activate the catabolism of stored carbohydrates (OMCL873). Genes encoding these enzymes are induced in representatives of all four kingdoms. Plants notably have alternative catabolic pathways that allow hydrolysis of starch to Glc by amylases (OMCL1389 and OMCL18208; Loreti et al., 2003) and ATP-independent catabolism of Suc by Suc synthase (OMCL855; Ricard et al., 1998). A complete set of starch-degrading enzymes, including α-amylase, β-amylase, debranching enzyme, and α-glucosidase, were induced in coleoptiles and germinating seeds of rice (Supplemental Fig. S5; Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009). However, amylases were not up-regulated strongly in all plants and organs (i.e. Arabidopsis seedlings, poplar roots), indicating that starch reserves may be unavailable or inaccessible. It was found previously that low-oxygen-sensitive seeds of cereal species, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), do not induce amylases under anoxia, in contrast to seeds of anaerobic germination-competent rice seeds (Guglielminetti et al., 1995). Neither Arabidopsis seedlings nor poplar roots induced amylases under oxygen deprivation; however, we cannot exclude amylase induction in seeds or other organs in these species.

Accompanying the induction of genes associated with the mobilization of sugars into glycolysis is the elevation of glycolytic enzyme and the decline in TCA cycle enzyme mRNAs in species of all four kingdoms (Fig. 3; Supplemental Figs. S4 and S5; Supplemental Table S6). Glycolytic enzymes are often encoded by gene families in eukaryotes with differentially regulated individual members. The reduced DEGs encoding TCA cycle enzymes and mitochondrial respiratory complex subunits were broadly conserved, although less evident in animals. mRNAs encoding pyruvate dehydrogenase kinase, which inhibits pyruvate dehydrogenase, thereby limiting pyruvate entry into the TCA cycle, were induced in studies of humans and

Figure 3. OMCL clusters of genes encoding proteins involved in glycolysis, fermentation, and the TCA cycle. Colors of bars correspond to proteins in the metabolic pathway at right. A single gene can be represented by multiple OMCL groups because of multisubunit enzyme complexes or large evolutionary differences in genes encoding the protein. Bright yellow, cluster has more than one induced DEG in that organism; light yellow, cluster has one induced DEG in that organism; bright blue, cluster includes more than one reduced DEG in that organism; light blue, cluster includes one reduced DEG in that organism; white, no DEG ortholog identified in that organism; gray, orthologous cluster/protein not identified in that organism. Extended data are shown in Supplemental Figure S4.

plants (OMCL985; Papandreou et al., 2006; Supplemental Fig. S6). Translational control of gene expression reduces TCA cycle enzyme synthesis in Arabidopsis, as demonstrated by the rapidly reversible shift of these mRNAs off of polysomes during low-oxygen stress (Branco-Price et al., 2008).

The cross-species analysis supports the hypothesis that plants enhance overall ATP production during an energy crisis by utilizing alternative glycolytic enzymes that consume pyrophosphate (PPi) instead of ATP. These include PPi-Fru-6-P phosphotransferase and pyruvate-orthophosphate dikinase (Bailey-Serres and Voesenek, 2008). This strategy was observed at all developmental stages evaluated for rice, which induced PPi-Fru-6-P phosphotransferase subunits, pyruvate-orthophosphate dikinase (OMCL946), and isoforms of the phospho*enol*pyruvate carboxykinase (OMCL2547; Huang et al., 2008), thereby improving energy usage in pyruvate metabolism under anoxia (Fig. 3; Supplemental Fig. S5). Genes encoding these PPi-dependent enzymes were more strongly induced in leaves of submergence-tolerant rice [compare M202(*Sub1*) with M202 in Supplemental Table S8]. Furthermore, a vacuolar PPi-dependent proton pump (OMCL1960), an alternative to ATP-dependent proton pumps, was preferentially induced in submergence-tolerant rice during submergence (Supplemental Fig. S5; Supplemental Table S8; Liu et al., 2010). As confirmed by mutational analyses of Suc synthase genes (Ricard et al., 1998; Bieniawska et al., 2007) and by decreasing PPi content of potatoes (*Solanum tuberosum*; Mustroph et al., 2005), these observations indicate a benefit of ATP-independent enzymes during low-oxygen stress in plants.

The meta-analysis also illuminated the evolutionarily conserved importance of trehalose-6-phosphate (T6P) metabolism (Fig. 3; Supplemental Fig. S4). T6P synthase (T6PS) transcripts (OMCL252) were induced in seven of 21 organisms under hypoxia, particularly in plants, fungi, and *E. coli*. Additionally, a T6P phosphatase was induced in plants and fungi (OMCL1954 and OMCL5544). T6P regulates glycolysis in yeast by inhibition of hexokinase (Blázquez et al., 1993). In plants, elevated T6P activates the starch-synthesizing enzyme ADP-Glc pyrophosphorylase, which subsequently limits glycolysis (Paul, 2007). In Arabidopsis, elevation of T6P increased growth by limiting the activity of the energy-sensing SNF1-related protein kinase 1 (SnRK1/AtKIN10/AtKIN11; Zhang et al., 2009). We found that submergence-tolerant rice induced three T6PS mRNAs to a greater extent than intolerant rice, raising the possibility that modulation of T6P may be relevant to the regulation of growth during submergence (Supplemental Table S8).

Conserved Induction of Stress Response Protein Genes

The transition to hypoxia and return to oxygenation promote the formation of ROS. The meta-analysis of ROS-regulated mRNAs found that HSPs were induced in both prokaryotes and eukaryotes (Vandenbroucke et al., 2008). Similarly, this study confirmed the induction of HSP and ROS network mRNAs in 16 and 10 organisms, respectively, including all the plants evaluated (Supplemental Fig. S7A). In addition to HSP mRNAs, heat shock TFs were DEGs in plants and yeast. Consistent with a functional role of HSPs during the stress, pre-heat-stressed Arabidopsis seedlings were more tolerant to anoxia (Banti et al., 2008, 2010). The prevalence of induced DEGs associated with the amelioration of ROS suggests that their detoxification is important for low-oxygen stress survival in diverse organisms (Supplemental Fig. S7B). Consistent with this, a correlation with the ability to ameliorate ROS and survival was reported for *Iris pseudacorus* (Monk et al., 1987) and rice (Ella et al., 2003). Along with ROS amelioration enzymes, plants uniformly induced mRNAs encoding the mitochondrial alternative oxidase (OMCL4174), which limits ROS formed by the mitochondrial electron transport chain upon reoxygenation (Rhoads and Subbaiah, 2007). Along with alternative oxidase, a conserved mitochondrial uncoupling protein (OMCL215) was an induced DEG in plants and animals (Supplemental Fig. S2). Finally, plasma membrane-localized respiratory burst oxidases that are implicated in signaling during hypoxia in plants and animals (Baxter-Burrell et al., 2002; Schroeder et al., 2009) were induced DEGs in Arabidopsis and *Caenorhabditis elegans* (OMCL290; Supplemental Table S6). The induction of HSPs and ROS network proteins in diverse organisms provides convincing evidence that the low-oxygen response includes components of a universal stress response (Feder and Hofmann, 1999; Swindell et al., 2007; Timperio et al., 2008).

Limited Conservation of Regulatory Proteins

Despite the conserved reconfiguration of primary metabolic enzymes and stress-associated protein mRNAs, there was limited conservation in the regulation of protein kinase (PK) and TF mRNAs (Supplemental Fig. S6). Exceptions included conservation in low-oxygen-induced mRNAs encoding MAPKs and MAPKKs (OMCL224 and OMCL302; Supplemental Table S6) as well as the CAMK-type kinases associated with energy sensing (i.e. OMCL212 and OMCL1019, including mammalian AMP-activated kinases [AMPKs; Fisslthaler and Fleming, 2009] and yeast SNF1 kinase [Hedbacker and Carlson, 2008]). Orthologs of the noncatalytic β-subunit of AMPK/SNF1 were induced in Arabidopsis, rice, and yeast (OMCL1419) but not in animals. By contrast, orthologs of the catalytic α-subunit were induced in rice (Os08g37800; SnRK1B) and the fungus *Trichoderma reesei* (OMCL1019). Although the Arabidopsis SNF1s *AtKIN10* and *AtKIN11* were not induced by hypoxia, the posttranslational regulation of these kinases mediates the sensing of energy status and gene regulation in leaf protoplasts (Baena-González et al., 2007). Interestingly, rice

SnRK1A (Os05g45420 and OMCL1019) is regulated by a calcineurin B-like-interacting protein kinase (CIPK15; Os11g02240 and OMCL39391) and is necessary to promote starch degradation during germination under low-oxygen conditions (Lee et al., 2009). The absence of close orthologs of CIPK15 in other plants may explain the unusual capacity of rice to germinate under anaerobic conditions. The AGC/RPS6K kinase family (OMCL318) also had DEGs in multiple organisms, including induced ribosomal S6-kinase members in humans (NP_037389), yeast (YMR104C), and higher plants (Supplemental Fig. S6; Supplemental Table S6). Although S6-kinase mRNAs are elevated in Arabidopsis seedlings by oxygen deprivation, they are not engaged with polysomes until reoxygenation (Branco-Price et al., 2008). A number of plant-specific PK mRNAs were differentially regulated by hypoxia in a species- or genotype-specific manner, including SHR5 receptor-like (OMCL123), Ser/Thr (OMCL139), and APK1A-like (OMCL5149) PKs (Supplemental Table S6). For example, some SHR5 receptor-like PKs had significantly stronger induction in leaves of submergence-tolerant rice, although the significance of the differential regulation remains unknown (Supplemental Table S8). The receptor-like PKs induced in *Chlamydomonas* were also largely distinct from those in higher plants (i.e. cluster OMCL39).

The meta-analysis also resolved differential expression of TF mRNAs in plants and other organisms (Supplemental Fig. S6). The regulation of mRNAs encoding factors involved in low-oxygen sensing was consistent with published reports (i.e. induction of *Sinorhizobium meliloti* and *Pseudomonas aeruginosa* FixJ/FixK TFs [Green et al., 2009; OMCL8812], *Mycobacterium tuberculosis* DosR TF [Sherman et al., 2001; OMCL2741], fungal Hap1/Rox1 TFs that measure cellular heme [Hon et al., 2003; OMCL4393 and OMCL1263], and MGA2 that regulates fatty acid desaturase [Jiang et al., 2001; OMCL6345] and no induction in posttranscriptionally regulated TFs, including *E. coli* redox-sensing ArcA/ArcB system and FNR TFs [Shalel-Levanon et al., 2005; Malpica et al., 2006] and mammalian hypoxia-inducible factor-1α-subunit [Bruick, 2003]; Supplemental Table S6). Plants lack orthologs of bacterial, yeast, and animal TFs associated with direct or indirect low-oxygen sensing (Bailey-Serres and Chang, 2005). However, orthologs of the oxygen-dependent prolyl hydroxylases, which sense physiological decline in oxygen in animals (EGL9 homolog [OMCL2339]; Supplemental Table S6; Bruick, 2003), were induced DEGs in multiple plants (OMCL700; Vlad et al., 2007), although their function in low-oxygen responses in plants is unknown. The meta-analysis of plant DEGs recognized the prevalent induction of TFs encoding the ethylene-responsive factor (ERF; MCL50), MYB (MCL46), CCCH-type zinc finger (MCL767), WRKY (MCL92), and bZIP (MCL196) family members (Supplemental Fig. S6; Supplemental Table S6). Several of these TFs affect survival in low-oxygen or flooding stress (i.e. MYBs [Hoeren et al., 1998; Lee et al., 2007; Mattana et al., 2007], ERFs [Fukao et al., 2006; Xu et al., 2006; Hattori et al., 2009], and NAC [Christianson et al., 2009]). Despite conservation of some TF families between higher plants and *Chlamydomonas* (Supplemental Table S4), there was only limited induction of closely related TFs in the plant and algae species evaluated. These included a family of MADS TFs, which were induced in Arabidopsis, rice, and *Chlamydomonas* and also in humans and yeast (OMCL171), and an orphan TF family (OMCL7327) induced in Arabidopsis, rice, and *Chlamydomonas* (Supplemental Table S6). Overall, this survey indicates that the TFs that mediate responses to oxygen deprivation are evolutionarily new relative to the conserved cellular acclimations they regulate.

Evolutionarily Divergent Adaptive Responses That Mediate Oxygen Delivery

Multicellular animals and plants can alter development in response to low-oxygen stress to facilitate oxygen delivery. For example, mammals can adjust pulmonary respiration rate, increase development of capillaries in hypoxic tissues, and stimulate red blood cell formation, as evidenced by enrichment in DEGs encoding proteins associated with these processes (Supplemental Tables S6 and S7a; i.e. vascular endothelial growth factor [OMCL4003; Ferrara et al., 2003] and adrenomedullin [OMCL16253; Guidolin et al., 2008]). Plant adaptation to a semiaquatic environment includes the formation of gas-filled aerenchyma that connect aerial leaves with submerged stems and roots, elongation of underwater stems and leaf petioles, and the initiation of adventitious roots (Bailey-Serres and Voesenek, 2008). Both the development of aerenchyma and underwater elongation growth involve ethylene. Plant-specific DEGs included several genes associated with ethylene biosynthesis (i.e.1-amino-cyclopropane-1-carboxylate synthase [OMCL714] and 1-amino-cyclopropane-1-carboxylate oxidase [OMCL28112]) and perception (OMCL4190). The induction of members of the expansin (OMCL4264 and OMCL6686) and xyloglucan endotransglucosylase (OMCL1722 and OMCL2239) gene families, which are involved in cell elongation growth, was also observed in all higher plants examined (Supplemental Table S5).

Conserved Versus Species-Specific Low-Oxygen-Responsive Genes of Plants

Our secondary objective was to recognize the core of plant-specific genes that respond to low-oxygen stress using Arabidopsis as a reference plant and model for reverse genetic analyses. Toward this goal, we examined data from seedling experiments that varied in duration or degree of oxygen deprivation, cell type, or genotype (Fig. 4; Supplemental Table S9, a and b). The analysis exposed strong similarity in the most highly

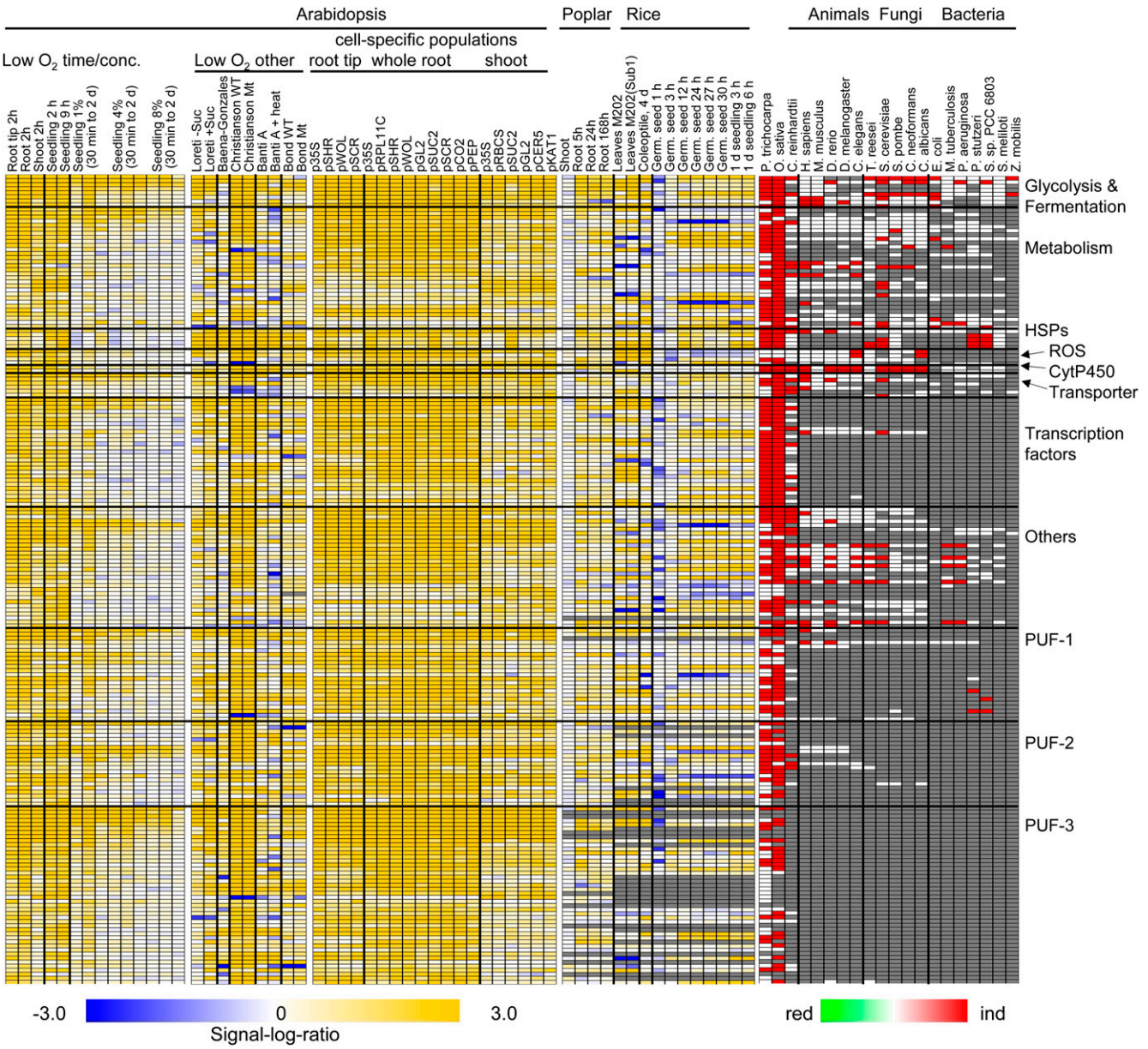

Figure 4. Low-oxygen-induced genes of Arabidopsis: expression across experiments, cell types, and orthologs in other organisms. The top 200 induced DEGs were selected from Supplemental Table S9. Each row represents one gene. Genes were manually grouped into functional categories. Left, Signal-log ratio values for genes across different plant experiments (Arabidopsis experiments, Loreti et al., 2005; Baena-González et al., 2007; Branco-Price et al., 2008; Bond et al., 2009; Christianson et al., 2009; Mustroph et al., 2009; van Dongen et al., 2009; Banti et al., 2010; Arabidopsis cell-specific populations, Mustroph et al., 2009, root tip, whole root, whole shoot; poplar experiments, Kreuzwieser et al., 2009; rice experiments, Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009; this study). Poplar and rice orthologs were identified by selecting the most highly expressed probe set either belonging to the same OMCL group as the Arabidopsis proteins or having a BLASTP similarity E value of 10^{-6} or less. conc., Concentration. Right, Orthologs in different organisms from plants (*P. trichocarpa, O. sativa, Chlamydomonas*), animals (*H. sapiens, M. musculus, D. rerio, D. melanogaster, C. elegans*), fungi (*T. reesei, S. cerevisiae, S. pombe, C. neoformans, C. albicans*), and bacteria (*E. coli, M. tuberculosis, P. aeruginosa, P. stutzeri, S. meliloti, Synechocystis* species PCC 6803, *Z. mobilis*). Orthologs were identified by a BLASTP similarity cutoff of $E \leq 10^{-6}$. Red, induced orthologs present in that organism; white, ortholog(s) not DEG(s); gray, no ortholog in that organism.

induced DEGs across Arabidopsis experiments, despite the variation in experimental parameters. The most highly induced DEGs of Arabidopsis were also induced in multiple cell-specific mRNA populations obtained by immunopurification of polysomes (Mustroph et al., 2009). The elevation of transcripts was less dramatic under less severe conditions and in shoots, as evidenced by lower induction of HSPs,

cytochrome P450s, and a number of TF mRNAs. A similar evaluation was performed with human data sets (Supplemental Fig. S8; Supplemental Table S10). mRNAs associated with anaerobic metabolism and signaling were induced in most root and shoot cell types. However, in contrast to plants, the survey of human cell types from different organs revealed considerable variation in induced DEGs of cells with distinct identity (Chi et al., 2006). The analysis also considered the reduced DEGs. As might be expected due to distinctions between cell types, the reduced mRNAs showed greater variation between cell-specific populations in both Arabidopsis and humans (Supplemental Figs. S9 and S10). In conclusion, the extent to which metabolism is reconfigured depends not only upon environmental parameters (Fig. 1) but also on genotype, organ, and cell type in plants and animals (Ellis et al., 1999; Chi et al., 2006; Mustroph et al., 2009).

To more accurately identify evolutionarily related genes with similar regulation in diverse species, we performed a BLASTP comparison of Arabidopsis proteins with whole proteomes of other organisms (Supplemental Table S9e). This provided a similarity score (E value) between proteins, allowing identification of proteins with strong to limited similarity (cutoff $E \leq 10^{-6}$). Of the 200 most highly induced DEGs of Arabidopsis, 75% had induced orthologs in rice leaves, coleoptiles, and/or germinating seeds (Fig. 4; Supplemental Table S9e). In poplar, where the response of root waterlogging was assessed, 50% of the Arabidopsis genes had induced orthologs. This value was only 15% for oxygen-deprived *Chlamydomonas*. Consistent with the OMCL analysis, the highly induced genes were associated with fermentation, Suc transport, ROS regulation, and heat shock response. Nonplant species possessed induced putative orthologs of 48 of the 200 most induced Arabidopsis genes, primarily in the metabolic and HSP categories (Supplemental Table S9e). The 30 most highly induced TFs were plant specific, with the exception of a C3H zinc finger and MADS domain family protein with modest similarity to TFs induced in other eukaryotes. The most conserved induced TF mRNAs of higher plants were zinc finger and LOB domain proteins. Highly induced TFs included group VII ERFs associated with the regulation of submergence responses (i.e. rice *Sub1A*, *Sub1C* [Os09g11460], and *ERF67* [Os07g47790]; poplar *ERF* [grail3_0003056501]; and Arabidopsis *ERF71* [At2g47520] and *ERF73* [At1g72360]). The conserved induction of group VII ERFs provides strong evidence for an ancestral role of this ERF TF subfamily in the survival of conditions with a low-oxygen component.

The analysis also identified a number of plant-specific induced DEGs encoding PUFs as annotated by the GO and/or SwissProt databases or based on the absence of a defined protein sequence motif in the PFAM database (Horan et al., 2008). Remarkably, nearly half (739) of the 1,860 low-oxygen-induced genes of Arabidopsis encode PUFs (e.g. *HUP* genes). Of these, 89% (659) have one or more putative ortholog in other plant species, including 58% (432) that are induced by one or more conditions with a low-oxygen component (Supplemental Fig. S11; Supplemental Table S9e). It is notable that 89 of the 200 most highly induced genes of Arabidopsis encode PUFs, most of which have orthologs limited to higher plants (Fig. 4). Examples of plant-specific hypoxia-responsive PUF families are universal stress proteins (OMCL2826 and OMCL3100), phloem-associated proteins (OMCL6676), and wound-responsive proteins (OMCL7410; Fig. 4; Supplemental Table S9c).

To investigate the role of proteins that lack a functionally defined protein domain in stress survival, mutants were established for 11 PUFs coexpressed with enzymes essential to anaerobic metabolism such as *ADH1* (Fig. 5A) and five genes coexpressed with other stress-induced proteins (Table I). These genes, designated as *HUPs*, varied in number of gene family members, predicted subcellular location, presence versus absence in other plants, and cell-specific pattern of expression (Fig. 5B; Supplemental Table S9). Comparison of the survival of homozygous mutant seedlings and wild-type (ecotype Columbia [Col-0]) seedlings confirmed that ectopic overexpression of *HUP* cDNAs, by use of the nearly constitutive 35S promoter, significantly modified the tolerance of prolonged oxygen deprivation in 12 of the 16 genes evaluated, including four Arabidopsis-specific genes. Overexpression of *HUP6*, *HUP9*, *HUP17*, *HUP23*, *HUP32*, *HUP41*, *HUP42*, and *HUP54* altered tolerance after 8 h of stress (Fig. 5C; Supplemental Fig. S12), whereas *HUP6*, *HUP9*, *HUP17*, *HUP29*, *HUP36*, *HUP37*, *HUP41*, and *HUP44* overexpressors showed altered tolerance after 12 h of stress (Supplemental Fig. S12). This confirmed that genes in several coexpression networks influence low-oxygen endurance. However, only some of the HUP overexpression lines were affected in low-oxygen survival. This could be due to a number of reasons. For example, a complex network may regulate the stress response, with products of most genes acting as modifiers because of redundancy in gene function and pathways. Alternatively, the use of the nearly constitutive 35S promoter may be ineffective if the level, timing, location, and conditional nature of gene expression are important parameters in determining the response to a transient stress. Interestingly, overexpression of *HUP6*, *HUP32*, and *HUP41* markedly reduced seedling tolerance of low oxygen, with the most dramatic decline observed in *HUP41* overexpressors (Supplemental Fig. S12K). The reduction in tolerance as a result of ectopic expression may indicate that these genes are critical in counterbalancing aspects of the stress response, such as carbohydrate consumption or ROS production (Fukao and Bailey-Serres, 2004). By contrast, overexpression of *HUP9* and *HUP17* was correlated with enhanced tolerance in independent transgenic lines, consistent with constitutive accumulation of the protein (Supplemental Table S11). T-DNA insertion homozygotes evaluated for six of the *HUPs* were altered in stress survival (Sup-

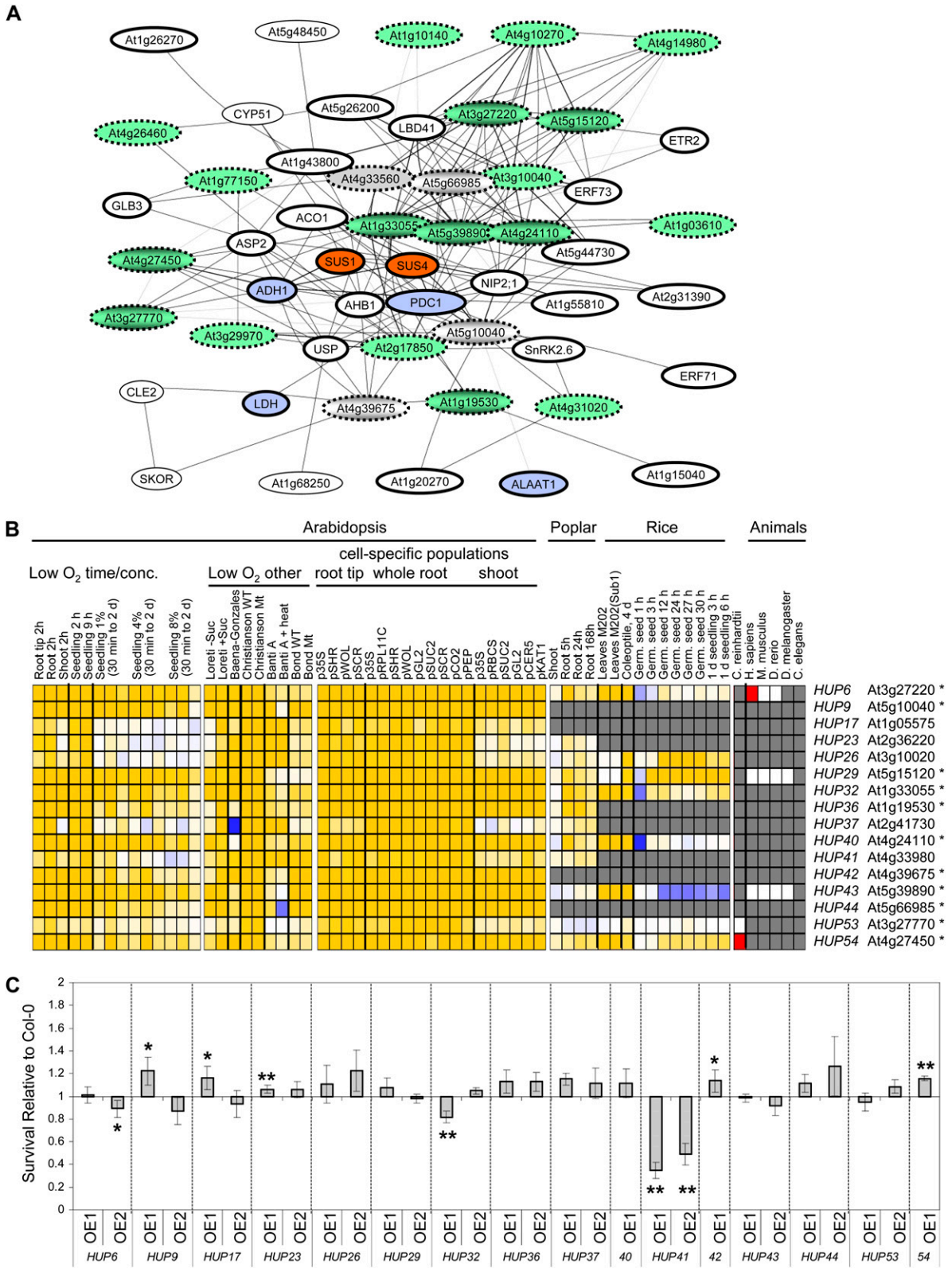

Figure 5. *HUP* genes coregulated with genes associated with anaerobic metabolism modify stress survival. A, Gene coexpression network for key enzymes associated with Suc catabolism and fermentation from the analysis of Ma et al. (2007). ACO1, 1-Aminocyclopropane-1-carboxylate oxidase (At2g19590); ADH1, alcohol dehydrogenase (At1g77120); AHB1, Arabidopsis hemoglobin 1 (At2g16060); ALAAT1, Ala aminotransferase (At1g17290); ASP2, Asp aminotransferase (At5g19550); CLE2, clavata 3/ESR related (At4g18510); CYP51, cytochrome P450 (At1g11680); ERF71 and ERF73, ERF group VII family TFs

Table I. *HUP genes analyzed for function in low-oxygen survival*

Gene Name	Arabidopsis Genome Initiative Locus	Ortholog(s) in Plants	Family Members[a]	Molecular Mass	Coexpressed Genes[b]
				kD	
HUP6	At3g27220	Rice, poplar	1_1_1	48.5	Anaerobic metabolism
HUP9	At5g10040	None	2_1_1	9.8	Anaerobic metabolism
HUP17	At1g05575	None	2_2_1	9.5	*ERFs*, *WRKYs*
HUP23	At2g36220	Poplar[c]	2_2_1	29.0	*PP2C*, *ERF4*
HUP26	At3g10020	Rice, poplar[c]	1_1_1	16.7	*HSPs*, *SnRK2*, *T6PS*
HUP29	At5g15120	Rice, poplar	5_2_1	33.0	Anaerobic metabolism
HUP32	At1g33055	Rice, poplar	1_1_1	7.5	Anaerobic metabolism, *ETR2*, *ZF*
HUP36	At1g19530	Poplar	1_1_1	13.7	Anaerobic metabolism
HUP37	At2g41730	Poplar[c]	2_2_2	13.3	*ACS2*, *NAC*, ROS response
HUP40	At4g24110	Rice, poplar[c]	1_1_1	28.6	Anaerobic metabolism
HUP41	At4g33980	Poplar[c]	1_1_1	24.6	*PDC2*, *ABF1*, *APRR5*, *ZF*
HUP42	At4g39675	None	1_1_1	8.2	Anaerobic metabolism
HUP43	At5g39890	Rice, poplar	19_7_1	30.8	Anaerobic metabolism
HUP44	At5g66985	None	1_1_1	9.4	Anaerobic metabolism
HUP53	At3g27770	Rice[c], poplar[c]	8_1_1	36.7	Anaerobic metabolism, *KCO1*
HUP54	At4g27450	Rice, poplar	5_2_2	27.6	Anaerobic metabolism

[a]Number of Arabidopsis genes with 35% or greater, 50% or greater, or 70% or greater amino acid sequence identity from Horan et al. (2008). [b]Coexpression network based on data of Ma et al. (2007) and Horan et al. (2008). Anaerobic metabolism coexpressed genes include one or more of the following: *ADH1*, *PDC1*, *SUS1*, *SUS4*, and *HB1*. *ABF1*, Abscisic acid-responsive element-binding factor 1; *ACS2*, 1-amino-cyclopropane-1-carboxylate synthase 2; *APRR5*, pseudoresponse regulator 5; *ERF*, ethylene responsive factor; *ETR2*, ethylene triple response 2; *HSPs*, heat shock proteins; *KCO1*, potassium channel 1; *NAC*, NAC domain TF family; *PP2C*, protein phosphatase 2C; *SnRK2*, SNF1-related protein kinase 2; *T6PS*, trehalose-6-phosphate synthase; *WRKY*, WRKY TF family; *ZF*, zinc finger protein. [c]Putative ortholog(s) identified but not induced by low oxygen (Supplemental Table S9e).

plemental Fig. S12), but these mutants had limited impact, possibly due to gene redundancy or incomplete gene disruption. Notably, the distribution and induction of *HUP* orthologs in the species evaluated was variable. *HUP9*, *HUP17*, *HUP42*, and *HUP44* were Arabidopsis or Brassicaceae specific; *HUP23*, *HUP36*, *HUP37*, and *HUP41* orthologs were present and induced in poplar; *HUP29* was present and induced in Arabidopsis, poplar, and rice; and *HUP54* was present and induced in higher plants and *Chlamydomonas*. These analyses confirm that PUFs with both broad and limited distribution within the plant kingdom can contribute to the survival of low-oxygen stress.

CONCLUSION

Here, the transcriptomic reconfiguration by low-oxygen stress in 21 organisms from four kingdoms was systematically evaluated to identify conservation and specificity in stress responses in plants. The grouping of proteins into OMCL clusters and recognition of protein families enriched in DEGs confirmed both broadly conserved and kingdom-specific characteristics in carbohydrate catabolism and fermentation. The carbohydrate catabolic pathways characteristic of higher plants contrasted with alternative metabolic pathways present in *Chlamydomonas* and some microorganisms. In addition to core metabolic reconfigurations that enhance substrate-level ATP production and NAD^+ regeneration, many species displayed increases in mRNAs encoding HSPs and enzymes associated with ROS networks as well as decreases in mRNAs encoding proteins associated with ribosome biogenesis and cell wall synthesis. Despite these conserved responses, the components of signal transduction pathways and transcriptional regulators were markedly distinct across organisms, indicating independent evolution of the regulatory networks at the kingdom and phylum levels. The comparison of the low-oxygen transcriptomes obtained with diverse experimental

Figure 5. (*Continued.*)
(At2g47520 and At1g72360); ETR2, ethylene response receptor (At3g23150); GLB3, Arabidopsis hemoglobin 3 (At4g32690); LBD41, LOB domain family protein (At3g02550); LDH, lactate dehydrogenase (At4g17260); NIP2;1, NOD26-like intrinsic protein (At2g34390); PDC1, pyruvate decarboxylase 1 (At4g33070); SKOR, Shaker family potassium ion channel (At3g02850); SnRK2.6, At4g33950; SUS1 and SUS4, Suc synthases (At5g20830 and At3g43190); USP, universal stress protein (At3g03270). Induced genes are indicated by thick ellipses; fill colors correspond to Figure 3. *HUPs* are indicated by dashed ellipses in gray (limited to Arabidopsis and near relatives) or green (present Arabidopsis, poplar, and rice) ellipses with genes targeted in mutant analyses shaded. B, Low-oxygen-induced *HUPs* of Arabidopsis: expression across experiments, cell types, and orthologs in other plants. conc., Concentration. Data are from Figure 4. Genes indicated with asterisks are present in the coexpression network shown in A. C, *HUP* mutant seedling survival of 8-h oxygen deprivation in dim light. Seedling viability was compared with Col-0 seedlings grown on the same plate. Error bars represent SD ($n = 3$). Asterisks indicate significant differences in survival of mutant relative to Col-0 (* $P \leq 0.1$, ** $P \leq 0.05$).

systems for three land plants (Arabidopsis, poplar, and rice) and across cell types of the root and the shoot of Arabidopsis exposed a large degree of conservation in response, extending to regulatory proteins. This study also found that the low-oxygen-induced genes encoding PUFs (e.g. *HUP* genes) of Arabidopsis were primarily limited to higher plants, in contrast to genes associated with reconfiguration of metabolism and general stress tolerance. Phenotypic evaluation of mutants overexpressing HUPs confirmed a role for these poorly characterized proteins in low-oxygen survival. We propose that these phylogenetically limited proteins act as modifiers to fine-tune responses to low-oxygen stress, although further investigation is necessary to determine their precise function. This study demonstrates both uniformity and distinction in the response to low-oxygen stress across the biological kingdoms, highlighting the evolution of regulatory networks and acclimation responses unique to higher plants.

MATERIALS AND METHODS

Data Set Collection and Analysis

Low-oxygen response expression data were obtained for 21 organisms from databases and publications: Arabidopsis (*Arabidopsis thaliana*; Branco-Price et al., 2008; van Dongen et al., 2009; Mustroph et al., 2009), rice (*Oryza sativa*; Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009), poplar (*Populus trichocarpa*; Kreuzwieser et al., 2009), *Chlamydomonas reinhardtii* (Mus et al., 2007), *Homo sapiens* (Kim et al., 2006; Chen et al., 2008; Guimbellot et al., 2009), *Mus musculus* (Greijer et al., 2005; Allen et al., 2006; Hu et al., 2006), *Danio rerio* (van der Meer et al., 2005; Marques et al., 2008), *Drosophila melanogaster* (Liu et al., 2006; Azad et al., 2009), *Caenorhabditis elegans* (Shen et al., 2005), *Trichoderma reesei* (Bonaccorsi et al., 2006), *Saccharomyces cerevisiae* (Linde et al., 1999; Lai et al., 2005; Protchenko et al., 2008), *Schizosaccharomyces pombe* (Todd et al., 2006), *Candida albicans* (Setiadi et al., 2006), *Cryptococcus neoformans* (Chun et al., 2007), *Escherichia coli* (Salmon et al., 2003; Kang et al., 2005; Covert et al., 2008), *Mycobacterium tuberculosis* (Park et al., 2003), *Pseudomonas aeruginosa* (Alvarez-Ortega and Harwood, 2007), *Pseudomonas stutzeri* (Dou et al., 2008), *Sinorhizobium meliloti* (Becker et al., 2004), *Synechocystis* species PCC 6803 (Summerfield et al., 2008), and *Zymomonas mobilis* (Yang et al., 2009). Additional Arabidopsis and human data sets evaluated responses across experimental conditions and cell types (Arabidopsis, Loreti et al., 2005; Baena-González et al., 2007; Bond et al., 2009; Christianson et al., 2009; Banti et al., 2010; human, Chi et al., 2006; Mense et al., 2006; Irigoyen et al., 2007; Ricciardi et al., 2008; Calzado et al., 2009; Fang et al., 2009; E-MIMR-644 [http://www.ebi.ac.uk/microarray-as/ae/], GSE15583 [http://www.ncbi.nlm.nih.gov/geo]). For the rice leaf samples, cv M202 and the *Sub1* introgression line M202(*Sub1*) were grown for 14 d and submerged for 1 d in a greenhouse exactly as described by Fukao et al. (2006). Total RNA was extracted from aerial tissue (Fukao and Bailey-Serres, 2008), and two independent biological replicate hybridizations to Rice Genome Arrays (GeneChip; Affymetrix) were performed using the manufacturer's protocol. These data are available from the Gene Expression Omnibus database as accession GSE18930.

Genes with a significant change in steady-state mRNA abundance in response to treatments were identified as follows. If Affymetrix CEL files were available, the data were reanalyzed by use of the R program (http://cran.at.r-project.org/) and Bioconductor package (http://www.bioconductor.org/; Gentleman et al., 2004). GC-corrected robust multichip average normalization was employed for the rice data sets, and the robust multichip average normalization was used for all other organisms. MAS5 present calls were obtained to remove probe sets that did not contain a P call in all replicates of at least one sample set. The LIMMA package was used for pairwise comparisons of stress treatment versus control and to obtain the signal-log ratio (Smyth, 2004). The Benjamini and Hochberg (1995) method was selected to adjust *P* values for multiple testing and to determine false discovery rates. In other cases, data were obtained from publications (Supplemental Table S1). To identify DEGs, a 2-fold change value was used for the initial selection. If fewer than 50 DEGs were identified for one organism, the change criterion was relaxed to 1.8-fold. Gene lists were further refined based on the robustness of data using a false discovery rate of 0.01. In some cases, other criteria were used due to the array setup or data availability (Supplemental Table S1). If multiple data sets were available, up to three from one organism were analyzed. Gene annotations were transformed into RefSeq accession numbers from the National Center for Biotechnology Information or another basic annotation system. Affymetrix (https://www.affymetrix.com/support) and PubMed (www.ncbi.nlm.nih.gov) data were used for annotation conversions.

BLAST Analysis of DEGs

FASTA protein sequences of whole proteomes were obtained: *C. albicans*, http://www.candidagenome.org/download/sequence/Assembly21/current/; *S. pombe*, http://www.sanger.ac.uk/Projects/S_pombe/protein_download.shtml; *S. cerevisiae*, http://downloads.yeastgenome.org/sequence/genomic_sequence/; *T. reesei*, http://genome.jgi-psf.org/Trire2/Trire2.download.ftp.html (Martinez et al., 2008); *Chlamydomonas*, http://genome.jgi-psf.org/Chlre3/Chlre3.download.ftp.html (Merchant et al., 2007); Arabidopsis, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR8_blastsets/; rice, http://rice.plantbiology.msu.edu/data_download.shtml; poplar, http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.download.ftp.html (Tuskan et al., 2006); and all other organisms, ftp://ftp.ncbi.nih.gov/genomes/.

An OMCL analysis of protein sequences was performed to identify homologous proteins as described by Li et al. (2003). First, an all-against-all BLASTP comparison was performed (Altschul et al., 1990). A *P* value of 10^{-5} or less was used for similarity cutoff. A similarity matrix was created based on *P* values, which was normalized for within- and between-species comparisons. Based on this similarity matrix, proteins were grouped with their homologs/orthologs by use of the Tribe-Markov clustering algorithm (Enright et al., 2002). Clusters of related proteins were formed with an inflation factor of 1.2 (clusters labeled with OMCL). Since this analysis resulted in a large number of small clusters, we additionally performed an MCL analysis of unnormalized *P* values according to Vandenbroucke et al. (2008) using an inflation factor of 1.5 to generate larger clusters, particularly to resolve regulatory proteins (clusters labeled with MCL). The resulting clusters are presented in Supplemental Table S5.

GO and PUF Assignment of Genes

GO annotations were obtained from public databases. First, all protein identifiers were transformed into UniprotKB identifiers by use of the ID Mapping feature of the Web site http://www.uniprot.org/. Then, all associated GO terms were obtained for the Uniprot identifiers using GOA files for each organism (ftp://ftp.ebi.ac.uk/pub/databases/GO/goa/proteomes/). For some organisms, GO annotations were directly obtained from organism-specific Web sites (http://genome.jgi-psf.org [*Chlamydomonas*, poplar, *T. reesei*], www.arabidopsis.org [Arabidopsis], and http://rice.plantbiology.msu.edu/ [rice]). GO enrichment analysis was performed with the GOHyperGall function according to Horan et al. (2008). Arabidopsis genes encoding PUFs were recognized based on Horan et al. (2008). Additionally, similarity to proteins by SwissProt (ftp://ftp.ebi.ac.uk/pub/databases/uniprot/current_release/knowledgebase/complete/uniprot_sprot.fasta.gz) was used to recognize PUFs in Arabidopsis and other organisms. Proteins were defined as "unknown" if the similarity E value to any protein of the SwissProt collection was lower than 10^{-6}.

BLAST Search for Related Arabidopsis Genes

For the stress-induced and -repressed genes, we performed BLAST analyses of Arabidopsis protein sequences against whole proteomes of the other 20 organisms (see above). The "blastall" function and a similarity E value cutoff of 10^{-6} or less were used to identify putative homologs/orthologs, with stress-induced or -repressed genes preferred over noninduced or nonrepressed genes, respectively, even if a noninduced or nonrepressed gene was more similar. The best of any hit for each Arabidopsis protein was selected.

Production and Stress Treatment of Arabidopsis Transgenics

p35S:HF, created from the binary T-DNA vector pPZP111 that contains a 1,343-bp cauliflower mosaic virus 35S promoter followed by the tobacco

mosaic virus Ω5′ untranslated leader, an N-terminal His_6-FLAG tag, and a 3′ NOS terminator (Zanetti et al., 2005), was modified to create two Gateway-compatible vectors: p35S:HF-GATA and p35S:GATA-HF for N- and C-terminal epitope tagging, respectively. The N-terminal tag is $M(H)_6(G)_3$DYKDDDDK $(G)_7$, the C-terminal tag is $(G)_7$DYKDDDDK$(G)_3(H)_6$, and GATA is the *att1-cmR-ccdb-att2* Gateway cassette in reading frame A (Invitrogen). Full-length cDNAs were obtained from the Arabidopsis Biological Research Collection or by amplification from reverse-transcribed mRNA from low-oxygen-stressed seedlings using gene-specific primers for At1g05575, At1g19530, At1g33055, At2g36220, At2g41730, At3g10020, At3g27220, At3g27770, At4g24110, At4g27450, At4g33980, At4g39675, At5g10040, At5g15120, At5g39890, and At5g66985 (Supplemental Table S11). Full-length open reading frames were amplified by PCR and cloned into the pENTR/D-TOPO vector (Invitrogen). Epitope tagging was at the C terminus, unless the predicted protein had a hydrophobic C terminus. Following recombination, the sequences of the tag junction and coding region were verified by sequencing (Institute for Integrated Genome Biology Core Facility, University of California, Riverside). Col-0 ecotype transformation, transgenic plant selection, and establishment of single-copy insertion lines were as described previously (Zanetti et al., 2005; Mustroph et al., 2009). Protein expression was confirmed as described in Supplemental Materials and Methods S1. At least two independent transgenic lines were established for each *HUP* gene.

Seeds were propagated on Murashige and Skoog medium (0.43% [w/v] Murashige and Skoog salts, 1% [w/v] Suc, and 0.4% [w/v] phytagel, pH 5.75) in square petri dishes in a vertical orientation at 23°C with a 16-h-day (50 μmol photons m^{-2} s^{-1}) and 8-h-night cycle for 7 d (Branco-Price et al., 2008), and then seedlings were deprived of oxygen for 8 or 12 h or mock treated under dim light (0.15 μmol photons m^{-2} s^{-1}) as detailed previously (Branco-Price et al., 2008). Treatments commenced at the end of the light period. After treatment, samples were returned to the growth chamber for 3 d. Viability of overexpressor lines, knockout mutants (Supplemental Table S11), and Col-0 seedlings grown on the same plate was scored as follows: the numbers of nondamaged, damaged, and dead plants (scores 5, 3, and 1, respectively) were summed for each genotype. Nondamaged plants had green aerial tissue and no evident tissue damage, damaged plants had anthocyanin-pigmented or bleached cotyledons or leaves, and dead plants had dead aerial meristems. Root growth was attenuated in all genotypes after 8 h of stress. Viability of overexpressor and knockout lines was compared with that of Col-0, using Student's *t* test to identify significant differences between means of replicates ($n = 3$) of different genotypes.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. OMCL clusters for hypoxia-induced and hypoxia-reduced DEGs.

Supplemental Figure S2. OMCL clusters with DEGs in at least six organisms of the 21 organisms analyzed.

Supplemental Figure S3. OMCL clusters associated with ribosomes.

Supplemental Figure S4. OMCL clusters with genes involved in glycolysis, fermentation, and the TCA cycle.

Supplemental Figure S5. Genes involved in primary metabolism: comparison across different treatment conditions, stages, and genotypes in higher plants.

Supplemental Figure S6. OMCL clusters of protein kinases and transcription factors.

Supplemental Figure S7. OMCL clusters with genes associated with heat shock ROS networks.

Supplemental Figure S8. Induced DEGs in low-oxygen-stressed human cells: expression patterns across experiments and cell types.

Supplemental Figure S9. Low-oxygen-reduced DEGs of Arabidopsis: expression patterns across experiments, cell types, and orthologs in other organisms.

Supplemental Figure S10. Reduced DEGs in low-oxygen-stressed human cells: expression patterns across experiments and cell types.

Supplemental Figure S11. Species distribution of low-oxygen-induced PUFs.

Supplemental Figure S12. Phenotype data for transgenic Arabidopsis plants expressing low-oxygen-induced *HUPs*.

Supplemental Table S1. Selected organisms and microarray experiments evaluating low-oxygen responses used in this paper.

Supplemental Table S2. Compilation of low-oxygen-induced genes in 21 organisms from four kingdoms.

Supplemental Table S3. Compilation of low-oxygen-reduced genes in 21 organisms from four kingdoms.

Supplemental Table S4. OMCL clustering of proteomes.

Supplemental Table S5. Assignment of proteomes of 21 organisms to OMCL clusters.

Supplemental Table S6. OMCL clustering of hypoxia-induced and hypoxia-reduced genes.

Supplemental Table S7. GO enrichment analysis of hypoxia-induced and -reduced genes in multiple organisms.

Supplemental Table S8. DEGs between two genotypes of the rice variety M202.

Supplemental Table S9. Observation of experimental and cell type variations in gene expression of hypoxia-induced and -reduced genes from Arabidopsis.

Supplemental Table S10. Observation of experimental and cell type variations in gene expression of hypoxia-induced and -reduced genes from human.

Supplemental Table S11. Summary of PUFs that were studied by mutant analysis.

Supplemental Materials and Methods S1.

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