A Genome-Wide Analysis of the Effects of Sucrose on Gene Expression in Arabidopsis Seedlings under Anoxia[w]

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Exogenous sucrose (Suc) greatly enhances anoxia tolerance of Arabidopsis (*Arabidopsis thaliana*) seedlings. We used the Affymetrix ATH1 GeneChip containing more than 22,500 probe sets to explore the anaerobic transcriptome of Arabidopsis seedlings kept under anoxia for 6 h in presence or absence of exogenous Suc. Functional clustering was performed using the MapMan software. Besides the expected induction of genes encoding enzymes involved in Suc metabolism and alcoholic fermentation, a large number of genes not related to these pathways were affected by anoxia. Addition of exogenous Suc mitigated the effects of anoxia on auxin responsive genes that are repressed under oxygen deprivation. Anoxia-induced Suc synthases showed a lower induction in presence of exogenous Suc, suggesting that induction of these genes might be related to an anoxia-dependent sugar starvation. Anoxic induction of genes coding for heat shock proteins was much stronger in presence of exogenous Suc. Interestingly, a short heat treatment enhanced anoxia tolerance, suggesting that heat shock proteins may play a role in survival to low oxygen. These results provide insight into the effects of Suc on the anoxic transcriptome and provide a list of candidate genes that enhance anoxia tolerance of Suc-treated seedlings.

Plants have to cope with a continuously changing environment, which challenges their ability to adapt to a variety of stress conditions. Adaptation to adverse growing conditions can be achieved by a preexisting anatomical trait or physiological behavior. Alternatively, a rapid change in gene expression triggered by the stressful event leads to an adaptative response. A number of genes involved in plant adaptation to a variety of biotic and abiotic stresses have been identified in the past. This was often achieved by searching for genes with a deduced/putative function in a specific adaptative response on the basis of preexisting knowledge of a similar behavior in other living organisms. Among abiotic stresses, anaerobic conditions trigger a rapid change in gene expression, leading to the production of a set of proteins involved in the adaptative response to low oxygen (anaerobic polypeptides; Sachs et al., 1996). This response has been characterized in a number of plant species (Perata and Alpi, 1993; Vartapetian and Jackson, 1997; Visser et al., 2003), including maize (*Zea mays*), rice (*Oryza sativa*), Echinocloa, amphibious plants (*Acorus calamus*, *Iris pseudacorus*) and semiaquatic plants (*Rumex* sp.). The use of Arabidopsis (*Arabidopsis thaliana*) as a model plant is a recent addition to the list of plant genera used to study plant adaptation to low oxygen (Dolferus et al., 1997, 2003; Ellis et al., 1999). The response to anoxia appears to be distinct in the roots and shoots of Arabidopsis, and caution should be used to extend to shoots results obtained using roots as an experimental system (Ellis et al., 1999). Dolferus et al. (2003) manipulated the fermentative pathway in Arabidopsis by overexpressing genes involved in the fermentative metabolism, showing that overexpression of pyruvate decarboxylase genes enhanced survival under low oxygen, while increased expression of either lactate dehydrogenase or alcohol dehydrogenase did not affect hypoxia tolerance. The recent progress in genome sequencing and related postgenomic approaches opens a new era in the study of plant response to anaerobiosis. Prior to the availability of microarrays covering the entire genome, this technique allowed an interesting and innovative approach to study the response of Arabidopsis root cultures to low oxygen (5% O_2) using a microarray containing 3,500 cDNA clones (Klok et al., 2002). The authors identified 274 differentially expressed genes under hypoxia, involved in metabolism (including 16 genes known to encode anaerobic polypeptides), signal transduction, and transcription. Furthermore, 50% of genes that encode proteins of unknown function where found to be induced or repressed by lowering oxygen availability to 5% (Klok et al., 2002).

Exogenous sugar availability strongly enhances tolerance to anoxia (Vartapetian and Andreeva, 1986;

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Perata et al., 1992; Germain et al., 1997; for review, see Vartapetian and Jackson 1997). To obtain further insight into the role of sugars in plant adaptation to anaerobiosis, we used the Affymetrix whole genome ATH1 array (Redman et al., 2004) to characterize the response of Arabidopsis seedlings treated for 6 h under anoxia in presence or absence of exogenous Suc. We used anoxia to obtain a rapid anaerobic response and choose seedlings as they represent a very homogeneous plants material at a growth stage at which plants often experience low oxygen conditions (Perata and Alpi, 1993). Anoxia triggered a strong change in gene expression and revealed many new anoxia-modulated genes. Although changes of transcript level do not necessarily result in changes at the protein level or enzyme activities, and the interpretation of transcript profiling at the physiological level is thus speculative, the resulting picture of our microarray analysis highlights previously unknown features of the response to anoxia and provides clues about the role of Suc in the enhancement of anoxia tolerance.

RESULTS AND DISCUSSION

The Anoxic Response in Arabidopsis Seedlings

We tested the mRNA accumulation of known anaerobic proteins in a time-course experiment to determine the most suitable anaerobic time point for transcript profiling analysis. Arabidopsis seedlings treated under anoxia rapidly induce known anaerobic response genes (alcohol dehydrogenase, *ADH*; pyruvate decarboxylase, *PDC1* and *PDC2*; nonsymbiotic hemoglobin, *AHB1*), and this induction lasts up to 8 h under anoxia (Fig. 1A). This is likely related to the survival of the seedlings, since seedlings that were kept under anoxia for 24 h did not recover when transferred to aerobic conditions (see the forthcoming paragraph). An additional time course experiment indicated that the induction of these anaerobic genes is detectable within 40 min of oxygen deprivation (Fig. 1B). Kürsteiner et al. (2003) identified *PDC1* as the only anoxia-inducible *PDC* gene, while our results show that *PDC2* also is anoxia-inducible. This discrepancy may be related to the younger age of seedlings in our study.

The presence of exogenous Suc greatly enhanced anoxia tolerance; seedlings recovering from the anoxic treatment were enhanced (Fig. 2A), and the accumulation of anoxia-induced mRNAs was prolonged (*ADH*, *PDC1*; Fig. 2B). Interestingly, Glc did not substitute for Suc in enhancing anoxia tolerance (Fig. 2C). A 6-h anoxia treatment appeared to represent an appropriate time point to compare the effects of anoxia in presence/absence of exogenous Suc. This time point represents the molecular response to prolonged anaerobiosis, prior to the onset of anoxia-induced death. We therefore decided to treat 4-d-old seedlings for 6 h under anoxia in the absence/presence of exogenous Suc, with the aim of identifying genes involved in Suc-dependent anoxia tolerance.

Validation of GeneChip Results

Arabidopsis seedlings were treated under anoxia for 6 h and mRNA accumulation was analyzed using the Affymetrix whole-genome array (ATH1). To verify whether the GeneChip results were reliable, we validated some of the results by northern analysis. Empirical evaluation of the detection limit of northern analysis, when compared to the expression level value resulting from GeneChip experiments, indicates that an Affymetrix-normalized expression value of 300 is associated to a transcript level easily detected by northern analysis. We thus identified genes strongly induced by anoxia (fold change, ≥3; expression level, ≥300), and then selected 7 genes from that list. The selection includes genes coding for a putative ethylene responsive protein (*At3g11930*; *ER6-like*), a putative aquaporin (*At2g34390*; *AQP*), a putative fructokinase (*At2g31390*; *FK*), and four genes encoding unknown proteins (*At5g39890*, *At5g10040*, *At4g33560*, and *At3g27220*; see Supplemental Table I). The expression analysis of the selected genes indicated that all were induced by anoxia, as predicted by the GeneChip experiment (Fig. 3, A and B). With the exception of *ER6-like*, all transcripts were barely detectable under aerobic conditions. Induction by anoxia was rapid, with a clear increase in transcript level detected as

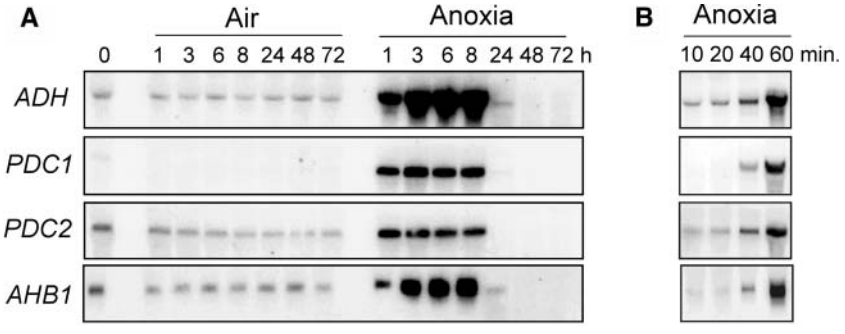

Figure 1. Pattern of mRNA accumulation for genes encoding known anaerobic proteins in Arabidopsis seedlings. A, Arabidopsis seeds were germinated under aerobic conditions for 4 d in liquid culture medium. Seedlings were kept under aerobic conditions (Air) or transferred to anoxia (Anoxia) for 1 to 72 h. B, Arabidopsis seeds were germinated under aerobic conditions for 4 d and subsequently transferred to anoxia for 10 to 60 min. RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (not shown). A representative experiment is shown.

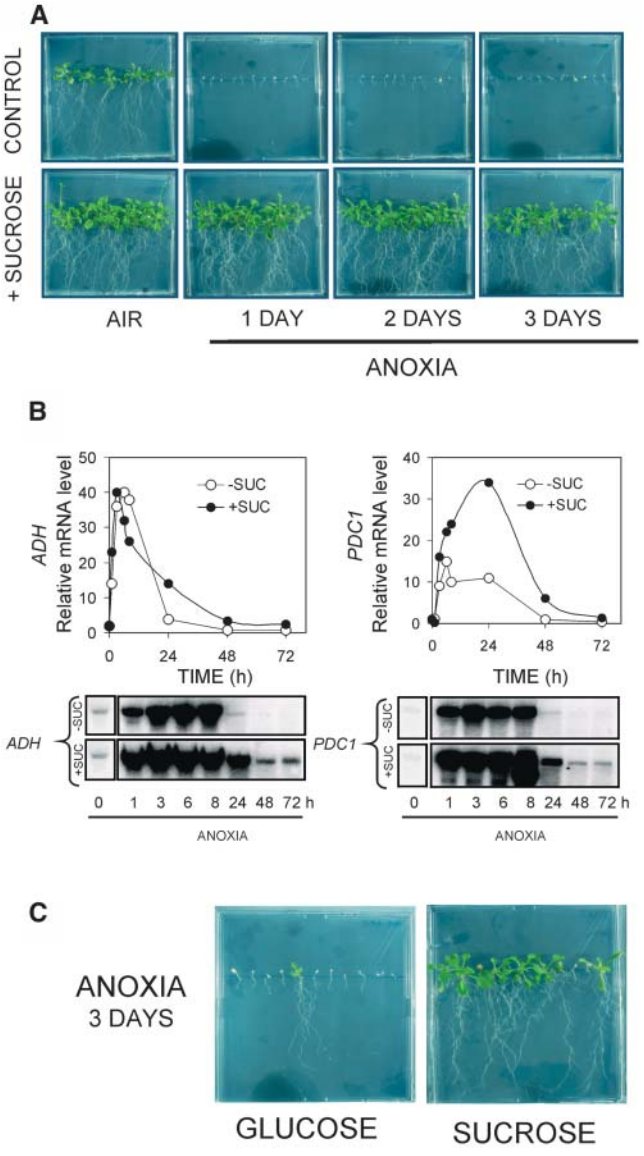

Figure 2. Effects of exogenous Suc on seedling survival and mRNA accumulation. A, Arabidopsis seeds were germinated under aerobic conditions for 4 d on vertical agar plates containing Murashige and Skoog medium (0.5×) in presence or absence of exogenous Suc (90 mM). When added, Suc was present throughout the experiment. Plates were subsequently transferred to anoxia for 1 to 3 d, and recovery of seedlings was observed after 2 additional weeks of aerobic growth in the light. B, Effect of exogenous Suc added during the anoxic treatment on the expression of *ADH* and *PDC1* genes. Arabidopsis seeds were germinated under aerobic conditions for 4 d in liquid culture medium and subsequently transferred to anoxia for 1 to 72 h. RNA was extracted, electrophoresed, and northern analysis carried out using gene-specific probes. The section reporting the results from the seedlings not treated with Suc was duplicated from Figure 1 to allow an easy comparison of the results. Equal loading was checked by reprobing with an rRNA probe (not shown). The quantitation of the mRNA in the northern analysis was performed and is reported in the graph above the respective northern-blot photograph. A representative experiment is shown. C, Effect of Glc and Suc on Arabidopsis survival after anoxia. Arabidopsis seeds were germinated under aerobic conditions for 4 d on vertical agar plates containing Murashige and Skoog medium (0.5×) in presence or absence of exogenous Suc or Glc (90 mM). Plates were subsequently transferred to anoxia for 3 d, and recovery of seedlings was observed after 2 additional weeks of aerobic growth in the light.

early as 40 min to 1 h of oxygen deprivation. *At5g10040* gene expression resulted in the accumulation of two transcripts, both induced by anoxia and showing slightly different expression timing.

The Anoxic Transcriptome in Presence and Absence of Exogenous Suc

The microarray results (see Supplemental Table I) showed that, under aerobic conditions, 13,247 probe sets (genes) give an Affymetrix call of "Present" (of a total of 22,746 probe sets present on the ATH1 GeneChip), while under anoxia the number of "Present" calls was slightly reduced (12,717). In the presence of exogenous Suc (anoxia + Suc), these figures changed only marginally (12,515). To select genes affected by anoxia, we considered only genes that showed a statistically significant and coincident change in call in the datasets from the 2 biological replicates, with a ±SD not exceeding 50% of the mean signal $\log_2$ ratio. Applying this cutoff, the number of genes induced by anoxia in absence/presence of Suc was 629 and 488, whereas the number of genes repressed by anoxia was 1,063 and 1,051, respectively. The genes affected by anoxia regardless of the presence/absence of exogenous Suc were 192 (induced) and 556 (repressed). Microarray expression data were then clustered using the MapMan software (Thimm et al., 2004). A proportion of the genes modulated by anoxia were genes of unknown function (40.4%, anoxia; 43.3% anoxia + Suc), whereas many of the other modulated genes belonged to various functional clusters (see Supplemental Table II, a and b).

Carbon Metabolism under Anoxia: Suc Repression of Suc Synthases

Anoxia triggers a dramatic change in the utilization of seed reserves, which are channeled into fermentative metabolism (Perata et al., 1997a). We used the microarray datasets to search for genes involved in lipid and carbohydrate metabolism to evaluate the effects of exogenous Suc on these pathways of primary importance for the establishment of the anaerobic metabolism and survival. Lipid is the primary carbon storage molecule in Arabidopsis seeds, constituting between 30% and 40% of dry weight, and the utilization of these reserves for energy production is of great importance for seedling growth (Eastmond et al., 2000). To obtain a global picture of the effect of anoxia on lipid and carbohydrate metabolism, we overlaid the statistically significant expression data on the pathways of lipid and sugar utilization, using the MapMan software (Thimm et al., 2004). Anoxia repressed several genes involved in the synthesis of fatty

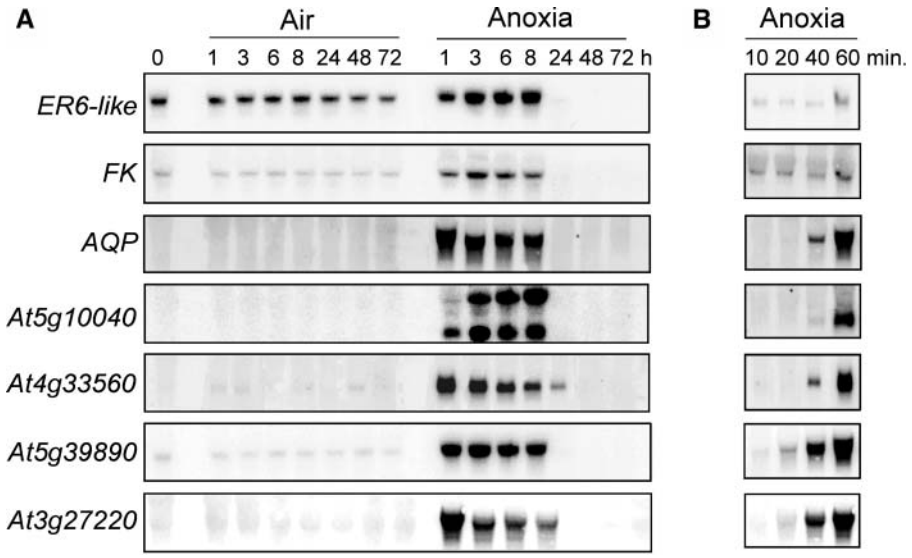

Figure 3. Validation of the anoxia-inducibility of genes selected on the basis of microarray analysis. A, Arabidopsis seeds were germinated under aerobic conditions for 4 d. Seedlings were kept under aerobic conditions (Air) or transferred to anoxia (Anoxia) for 1 to 72 h. B, Arabidopsis seeds were germinated under aerobic conditions for 4 d and subsequently transferred to anoxia for 10 to 60 min. RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (not shown). A representative experiment is shown.

acids (see Supplemental Table IIa). Genes coding for enzymes of the lipid degradation pathway were also repressed, mostly at the level of lipases, while the genes encoding β-oxidation and the glyoxylate cycle genes were unaffected or moderately induced by anoxia (see Supplemental Table II, a and b). Repression of lipases may avoid the build-up of fatty acids that cannot be metabolized further, as β-oxidation requires molecular oxygen. The ''anoxia'' datasets were compared with the ''anoxia + Suc'' datasets using the MAS 5.0 algorithm (see ''Materials and Methods'') to identify genes significantly modulated by the Suc treatment. The results indicated that the availability of Suc did not affect significantly genes involved in lipid metabolism.

Utilization of starch and Suc is of great importance for anoxia tolerance (Perata et al., 1998). An overview of the effects of anoxia on the pathways involved in carbohydrate metabolism is reported in Figure 4A. The microarray results highlight the strong induction of two Suc synthase genes (*At3g43190* and *At5g20830*), which suggests that a Suc-synthase pathway is active for Suc utilization under anoxia, as previously proposed for rice seedlings (Guglielminetti et al., 1995). Three putative invertases were also induced (*At5g11920*, *At3g27570*, and *At1g12240*) and the repression of a neutral invertase (*At1g35580*) was observed. Suc metabolism through the Suc synthase pathway offers several advantages as an energy-recovering pathway (Guglielminetti et al., 1995; Bologa et al., 2003), and it is notable that Glc cannot substitute for Suc as a carbon source supporting anoxia tolerance (Fig. 2C). Anaerobic induction of the two Suc synthase genes detected by microarray analysis has been previously observed (Baud et al., 2004). A gene encoding for a Suc-P-synthase (*At5g11110*) was also induced, suggesting that Suc may also be synthesized under anoxia, although this would be an energy consuming process (Guglielminetti et al., 1999). The pathway of starch degradation is moderately induced under anoxia. Taken together, these results indicate that in Arabidopsis seedlings, anoxia predominantly induces the genes required to degrade Suc, not starch.

We observed a generalized anoxic induction of transcripts encoding enzymes of glycolysis. Several glycolytic genes were induced by anoxia (Fig. 4A). Genes involved in alcoholic fermentation such as *ADH* (*At1g77120*), *PDC1* (*At4g33070*), and *PDC2* (*At5g54960*) showed a dramatic increase in expression, in agreement with Klok et al. (2002). A strong induction of two aminotransferases was also observed (see Supplemental Table II, a and b). The fermentative production of ethanol by the concerted action of pyruvate decarboxylases and alcohol dehydrogenase may be unable to consume the pyruvate accumulating as a consequence of the inactivity of the Krebs cycle. The induction of an Ala aminotransferase (*At1g17290*) gene and accumulation of the encoded enzyme may allow the conversion of the excess pyruvate to Ala (see Supplemental Table II, a and b). The production of Ala is indeed relevant in rice roots, reaching up to 1.2% of the dry weight after 24 h under anoxia (Reggiani and Bertani, 2003). The cytosolic Asp aminotransferase *ASP2* (*At5g19550*) was also strongly induced by anoxia (see Supplemental Table I). This enzyme initiates a cascade of metabolic reactions leading to fumarate functioning as an electron acceptor under anaerobic conditions (Pisarenko et al., 1995). A role for this enzyme in the posthypoxic recovery of isolated rat heart has been proposed (Pisarenko et al., 1995), while, to our knowledge, this hypothesis has not been tested in plants.

The respiratory activity of mitochondria is hampered by the absence of oxygen, but these organelles play an important role in the anoxic burst of cytosolic calcium (Subbaiah et al., 1998). Furthermore, anoxia exerts negative effects on plant mitochondria

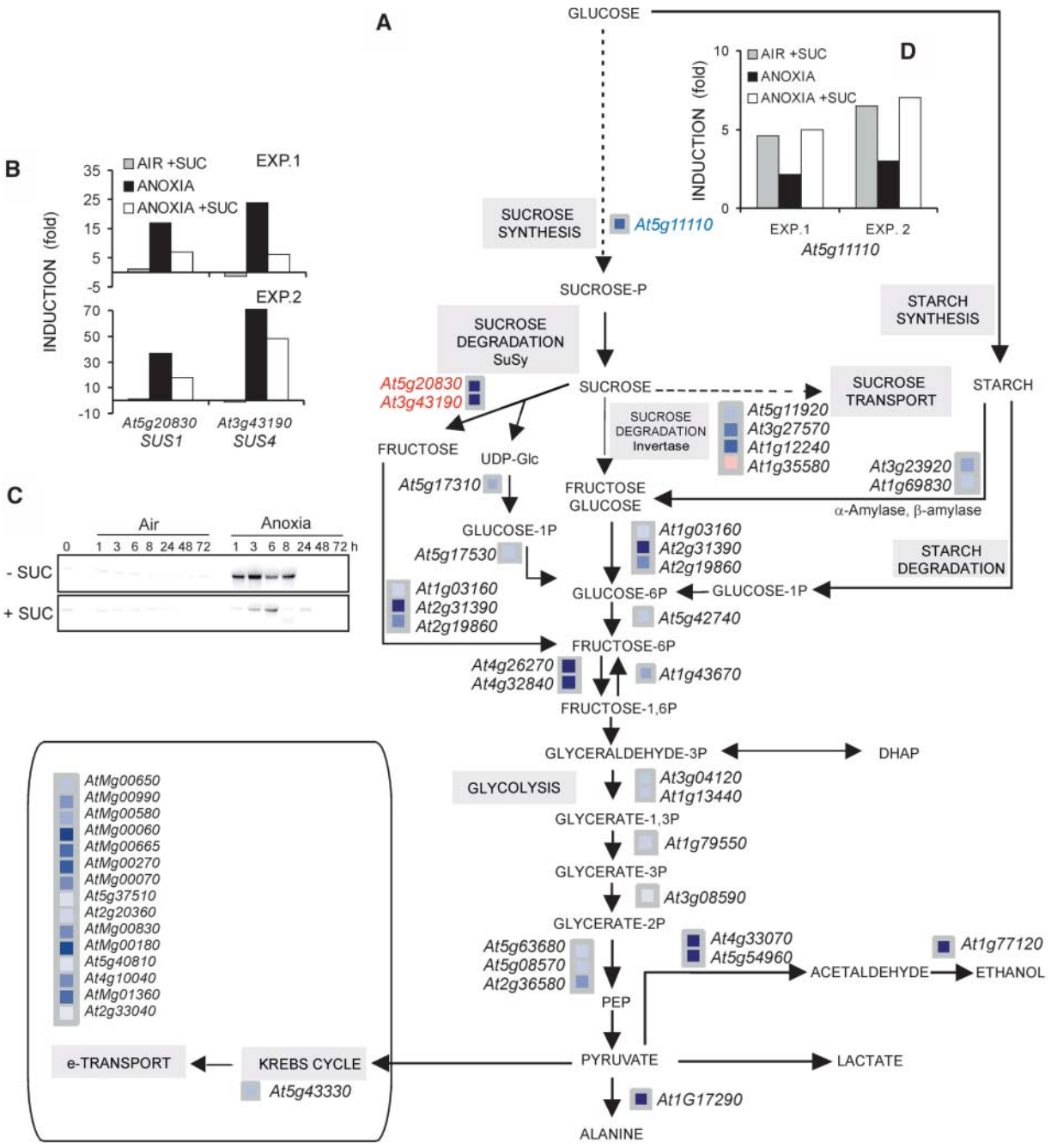

Figure 4. Effects of anoxia on carbohydrate metabolism, glycolysis, and mitochondrial respiration. A, Arabidopsis seeds were germinated under aerobic conditions for 4 d and subsequently transferred to anoxia for 6 h. Microarray data (averaged changes in transcript level from two biological replicates) were analyzed using the MapMan software. The output of the software is shown, with the genes involved in each metabolic step represented by a small square. A blue square (and blue shades) indicates a gene whose transcript level increased following the anoxic treatment. A red square (and red shades) indicates a gene whose transcript level decreased following the anoxic treatment. A color scale was used, in which a pale coloration represents a 1-fold change in expression, and the response saturates at a 4-fold change. Arabidopsis Genome Initiative code colored in blue/red indicates a stronger/reduced anoxia response, respectively, in the presence of exogenous Suc. B, Effects of Suc on the expression of two anoxia-inducible Suc synthase genes. Suc synthase genes significantly affected by exogenous Suc were identified by comparing the datasets (air versus air + Suc; air versus anoxia; air versus anoxia + Suc) by using the Microarray Analysis Suite 5.0 software algorithm. The fold-change measured in two independent experiments (EXP. 1 and EXP. 2) is shown. C, Time course of the expression of *SUS1* in presence/absence of Suc. RNA was extracted, electrophoresed, and northern analysis carried out using a gene-specific probe. Equal loading was checked by reprobing with an rRNA probe (not shown). A representative experiment is shown. D, Effects of Suc on the expression of a Suc-P-synthase gene (*At5g11110*). This Suc-P-synthase gene was identified by comparing the datasets (air versus air + Suc; air versus anoxia; air versus anoxia + Suc) by using the Microarray Analysis Suite 5.0 software algorithm. The fold-change measured in two independent experiments (EXP.1 and EXP. 2) is shown.

ultrastructure (Vartapetian et al., 2003). Interestingly, while the expression of most of the genes coding for proteins involved in the Krebs cycle is unchanged under anoxia, a large number of genes coding for proteins belonging to the mitochondrial electron transport (mostly NADH dehydrogenases) were induced by anoxia (Fig. 4A; Supplemental Table IIa). It is tempting to speculate that the low ATP level under anoxia may trigger up-regulation of key genes needed for energy production, including the mitochondrial electron transport. This response may be useless under anoxia (although the mitochondria may require an unknown length of time to become anoxic), whereas it may play a role for adaptation to hypoxia or flooding.

Remarkably, as far as the pathway shown in Figure 4A is concerned, the addition of Suc to the incubation media affected significantly only three genes. Expression of a Suc-P-synthase (*At5g11110*) was enhanced by Suc (AGI code colored in blue in Fig. 4A), whereas the mRNA level of two Suc synthases was lowered under anoxia in the presence of Suc (AGI code colored in red in Fig. 4A). Six distinct members are represented in the Suc synthase family of Arabidopsis (Baud et al., 2004). Two genes show anoxia-inducibility, namely *SUS1* (*At5g20830*) and *SUS4* (*At3g43190*). Neither of these two genes showed an induction in seedlings in response to Suc (Baud et al., 2004; Fig. 4B), but they appeared to be sugar-responsive when induced by anoxia (Fig. 4B). This result was confirmed in two independent experiments (Fig. 4B). A time course experiment confirmed that *SUS1* induction under anoxia was strongly reduced when seedlings were fed with exogenous Suc (Fig. 4C). It has been proposed that some anoxia-induced genes are modulated as a consequence of the anoxia-induced altered sugar status, while other genes are independently modulated by sugars or anoxia (Loreti et al., 2003). Our result suggests that induction of Suc synthase by anoxia might be, at least partially, due to an anoxia-induced starvation effect, as suggested by Koch et al. (2000) for maize Suc synthase *Sh1* gene, which is induced by anoxia as well as by sugar starvation. Suc-P-synthase (*At5g11110*) mRNA accumulation was increased under anoxia (Fig. 4D). The Suc-dependent enhanced expression of Suc-P-synthase (*At5g11110*) was observed under either aerobic or anaerobic conditions (Fig. 4D), indicating that the sensing mechanism responsible for the Suc-dependent modulation of this gene is operating also under anoxia.

Exogenous Suc Mitigates the Negative Effects of Anoxia on Genes Involved in Auxin Physiology

Besides the effects of Suc on the expression of Suc synthase genes (Fig. 4, B and C) and Suc-P-synthase (Fig. 4D), exogenous Suc affected the expression of other anoxia-modulated genes. A scatter plot of anoxia-modulated genes revealed that two group of genes were affected by exogenous Suc (Fig. 5A; see Supplemental Table III). A first group included genes whose anoxia-responsiveness was attenuated by exogenous Suc (Fig. 5A, red dots). A second group included genes showing a stronger response to anoxia in presence of Suc (Fig. 5A, blue dots). Genes coding for proteins of unknown function are largely represented in both groups of Suc-modulated genes (see Supplemental Table III).

As far as genes whose anoxia-inducibility was lowered by Suc, we observed repression of several genes involved in auxin physiology (see Supplemental Table III). We selected all the genes involved in auxin physiology represented on the GeneChip and showing a significant modulation by anoxia. The induction values, expressed as fold change, were plotted against the induction values observed under anoxia in presence of exogenous Suc, revealing that Suc effectively reduces the negative effects of anoxia on this gene cluster (Fig. 5B). The negative effects of anoxia on auxin-related genes are of interest and suggest that either auxin level or signaling is altered by anoxia. All these genes code for auxin-inducible transcripts, but little is known about the role of auxin signaling under anoxia or hypoxia, although a negative effect of anoxia on auxin-binding activities has been reported in rice (Mapelli and Locatelli, 1995).

Suc Enhancement of Anoxia-Inducibility of Heat Shock Proteins May Play a Role in Anoxia Tolerance

Genes coding for heat shock proteins (HSP) were highly represented in the group of genes showing Suc-enhanced response to anoxia (Fig. 5A, blue dots; see Supplemental Table III). We therefore decided to search for all genes coding for heat shock proteins represented on the GeneChip and showing a significant induction by anoxia. The induction values, expressed as fold change, were plotted against the induction values observed under anoxia in presence of exogenous Suc, revealing that several HSP genes were indeed up-regulated by Suc under anoxia (Fig. 5C). This positive effect cannot be ascribed to a better overall metabolism of seedlings treated with exogenous Suc, since the global effect of Suc on transcript level is negligible (see dotted line in Fig. 5C). The HSP genes most affected by Suc under anoxia are listed in Figure 5D. The anoxic-induction of several HSP genes was strongly enhanced by Suc, and this effect appeared to be anoxia-specific, since induction by Suc in air was modest (Fig. 5D). Experimental evidence has been published of heat-induction of anaerobic genes in maize seedlings (Russell and Sachs, 1989), and heat shock genes are induced by anaerobiosis in rice embryos (Mocquot et al., 1987). Some HSP are induced by hypoxia in animal systems, and a moderate heat shock results in resistance to hypoxia (Snoeckx et al., 2001). We tested whether treating Arabidopsis seedlings at 38°C (Queitsch et al., 2000) for 1.5 h, immediately before transfer to 6-h anoxia, resulted

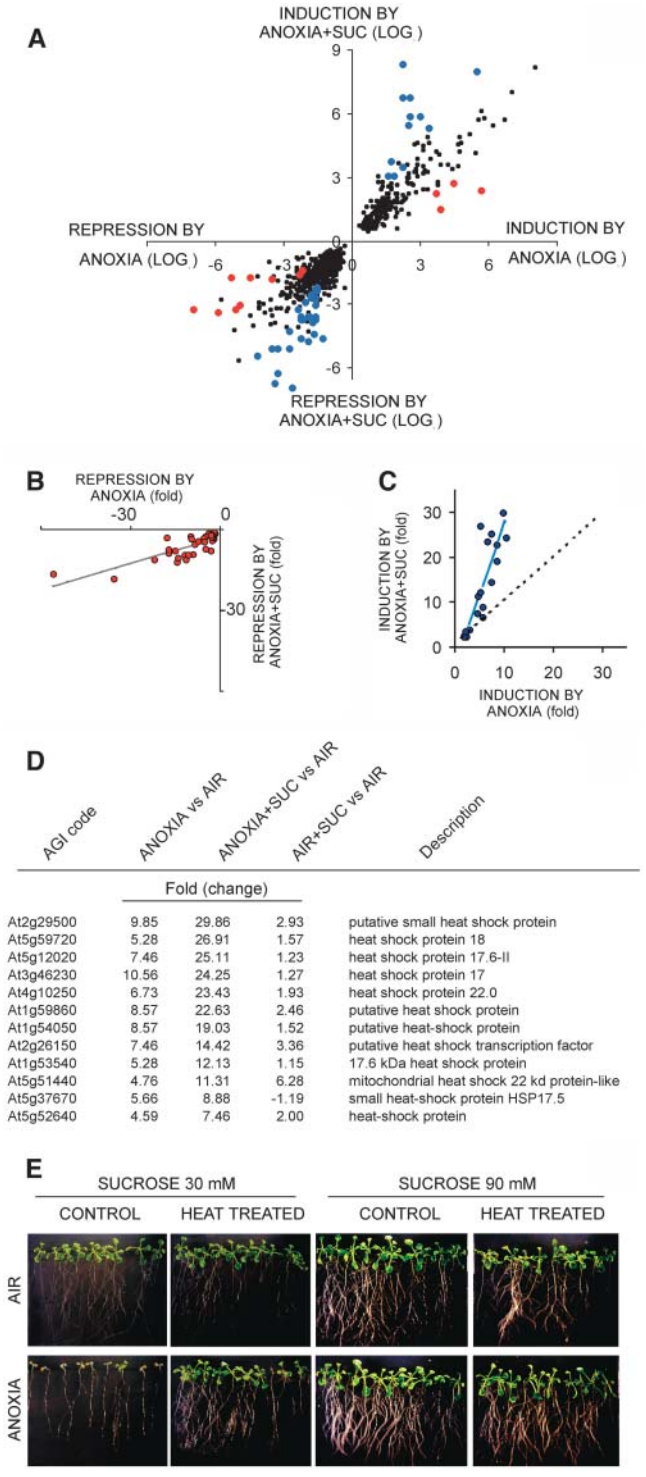

Figure 5. Effects of Suc on the anoxic transcriptome. A, Scatter plot showing the correlation between gene modulation under anoxia and under anoxia with exogenous Suc (ANOXIA + SUC). Expression data were filtered to select only genes showing a coincident change-call in both the replicates and for each experimental condition. Furthermore, only replicates whose ±SD of the averaged signal $\log_2$ ratio did not exceed 50% of the mean value were selected. Genes significantly affected by exogenous Suc were identified by comparing the datasets (anoxia versus anoxia + Suc) by using the Microarray Analysis Suite 5.0 software algorithm. Only genes showing a significant change call in both the replicates were selected and are shown as blue and red dots in the scatter plot. Blue dots identify genes whose anoxia-modulation was enhanced by Suc, while red dots identify genes showing a reduction of the anoxic response as a consequence of Suc addition to the incubation medium. B, Genes significantly repressed by anoxia and annotated as coding for proteins involved in auxin physiology were selected, and their change of expression (fold change) under anoxia was plotted against the anoxia + Suc fold change. C, Genes significantly induced by anoxia and annotated as coding for HSP were selected, and their change of expression (fold change) under anoxia was plotted against the anoxia + Suc fold change. As a reference for the overall effects of Suc on gene induction, the regression line of data reported in Figure 5A is shown as black dotted line. The data reported are mean of two biological replicates ±SD did not exceed 50% of the mean value. D, Effect of Suc, anoxia, and anoxia + Suc on the transcript level of genes coding for HSP. Only genes showing a clear response to presence of Suc under anoxia are listed. Data are expressed as average fold change from two biological replicates for each experimental condition. E, Effect of heat pretreatment on anoxia survival of Arabidopsis seedlings. One-week-old seedlings were used, to allow an easy visualization of the root system. Seedlings were treated at 38°C for 1.5 h immediately before the 6-h anoxia treatment. Exogenous Suc was added to the growing medium at the concentration shown in figure. A representative experiment is shown.

in enhanced tolerance to oxygen absence. Figure 5E shows that heat-treated seedlings survive better to anoxia, while 90 mM Suc, a concentration triggering enhanced HSP-gene transcript accumulation (Fig. 5, C and D) is enough to ensure survival to 6 h anoxia, as shown in Figure 2. These results suggest that the addition of Suc under anoxia may enhance the production of heat shock proteins. These proteins may act as chaperones, allowing the correct folding of proteins produced under anoxia.

Concluding Remarks

Transcript steady-state level detected by microarray analysis does not take into account the possible regulation of transcript translation efficiency (Fennoy and Bailey-Serres, 1995). However, whole-genome transcript profiling provides a significant amount of new information about the response of plants to anoxia at molecular level, which can be further studied using postgenomic approaches, including profiling of proteins.

Several of the anoxia-modulated genes whose expression is affected by Suc encode proteins of unknown function, and an effort to identify the function of these genes and their role in anaerobiosis tolerance requires the use of mutant lines for the genes of interest. A search in the mutant collections databases reveals that a putative knockout line is available for about 50% of the anoxia-modulated genes identified in the present work. A high-throughput screening of anoxia tolerance in these mutant lines is in progress and will hopefully lead to a more complete understanding of plant responses to anaerobiosis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia *glabra* was used in this study. Seeds were sterilized with diluted bleach (10-min incubation in 1.7% sodium hypochlorite, rinsing and washing in sterile water 7 times) and incubated in 2.5 mL of liquid growing media (Murashige and Skoog half-strength solution in 6-well plates). Plates were incubated in the darkness at 4°C for 2 d and finally transferred to 23°C for 4 d before the treatments. When used, sugars were added immediately before the beginning of the anaerobic treatments. An enclosed anaerobic work station (Anaerobic System model 1025; Forma Scientific, Marietta, OH) was used to provide an oxygen-free environment for seedling incubation. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to less than 10 μg mL^{-1} O_2 (according to the specifications provided by the manufacturer). High purity N_2 is used for purging the chamber initially and the working anaerobic gas mixture was N_2:H_2 proportioned at 90:10. Treatment with Suc (90 mM) was also performed in aerobic conditions. Suc was added immediately before the anoxic treatments. Two independent, replicated experiments were performed for each experimental condition. Each independent experiment consisted of four replicated seedling cultures pooled after RNA extraction. Vertical plates, used to evaluate anoxia tolerance, were prepared using solid growing media (Murashige and Skoog half strength solution, containing 1% agar). Vertical plates were transferred to light for postanoxic recovery (14-/10-h photoperiod at 150 μmol photons m^{-2} s^{-1}). When added, Suc or Glc was present throughout the experiment. Heat shock treatments were performed by transferring the plates containing the 7-d-old Arabidopsis seedlings to 38°C for 90 min immediately before the anoxic treatment.

Probe Design and Preparation

PCR primers were designed to amplify the most specific region inside the Affymetrix target region (sequence alignment was checked by the free Web interfaced software ClustalW, http://www.ebi.ac.uk/clustalw/). For the design of the primers, we used the free Web interfaced software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Oligonucleotides were purchased from MWG-Biotech (High Point, NC). Primer sequences for each gene are listed in the Supplemental Table IV. Poly(A^+) RNA was purified by Oligotex (Qiagen, Valencia, CA) from total RNA extracted from 4-d-old Arabidopsis seedlings incubated for 6 h under anoxic conditions. About 150 ng of purified poly(A^+) RNA was reverse transcribed with random primers by Improm-II (Promega, Madison WI) for 1 h at 42°C. PCR amplification on 15 ng cDNA (or 150 ng genomic DNA for intronless probed region) was performed with 400 nM specific primers and 2X PCR MIX (Promega). PCR conditions were as follows: 94°C for 2 min, 30 PCR cycles; 94°C for 45 s, primers annealing temperature (see Supplemental Table IV) 45 s; and 72°C for 45 s with a final extension of 8 min at 72°C.

RNA Isolation and Gel Blots

RNA extraction was performed by using the aurintricarboxylic acid method as previously described (Perata et al., 1997b). The amount of total RNA loaded per lane for electrophoresis was 20 μg. RNA was electrophoresed on 1% (w/v) agarose glyoxal gels and blotted on nylon membrane (BrightStar-Plus, Ambion, Austin, TX) by using the procedure suggested by the manufacturer. Membranes were prehybridized and hybridized using the northernMax-Gly kit (Ambion). Radiolabeled probes were prepared from gel-purified cDNAs by random primer labeling (Takara Chemicals, Shiga, Japan) with [α^{32}P]dCTP. Equal loading was checked by reprobing with an rRNA cDNA probe (not shown). RNA blots were scanned using a Cyclone Phosphoimager (Packard Bioscience, Perkin Elmer, Foster City, CA). mRNA level was quantified using the Optiquant software (Packard Bioscience, Perkin Elmer).

RNA Isolation, cRNA Synthesis, and Hybridization to Affymetrix GeneChips

Total RNA was extracted from the seedling samples, using the Ambion RNAqueous extraction kit (Ambion). RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix Arabidopsis ATH1 GeneChip arrays, according to the manufacturer's protocol. In brief, 10 μg of total RNA was used in a reverse transcription reaction (Ambion MessageAmp kit) to generate first-strand cDNA. After second-strand synthesis, double-strand cDNA was used in an in vitro transcription reaction to generate biotinylated cRNA. After purification and fragmentation, biotinylated cRNA was used for hybridization. The cRNA's quality was checked by hybridizations on Affymetrix Test 3 arrays. Hybridization, washing, staining, and scanning procedures were performed by Biopolo (University of Milano Bicocca, Italy) as described in the Affymetrix technical manual. Expression analysis via the Affymetrix Microarray Suite software (version 5.0;Affymetrix, Santa Clara, CA) was performed with standard parameters. Two independent, replicated experiments were performed for each experimental condition, and the output of the Affymetrix Microarray Suite software for each independent experiment was subjected to further analysis by using Microsoft Excel (Microsoft, Redmond, WA). Microarray datasets were deposited in a public repository with open access (accession no. GSE2133, http://www.ncbi.nlm.nih.gov/projects/geo/). Signal values (indicating the relative abundance of a particular transcript) and detection call values (indicating the probability that a particular transcript is present) were generated by Microarray Analysis Suite 5.0 software (Affymetrix). Probe pair sets (genes) called "Absent" were removed from subsequent analyses. Furthermore, genes with "Absent" for the detection value in the baseline data and "Decrease" for the change call were excluded from the list. Similarly, genes with "Absent" for the detection call in the experimental data and "Increase" for the Change value were also excluded from the list. Differences in transcript abundance, expressed as fold change, were calculated using the Microarray Analysis Suite 5.0 software change algorithm. Fold change was assumed to be correct only if the corresponding "change call" indicated a significant change ("I," Increase; "D," Decrease, generated by Microarray Analysis Suite 5.0 software). Expression data were filtered to select only genes showing a coinciding change-call in the two biological replicates samples for each experimental condition. Furthermore, only gene replicates with a $\pm$SD that did not exceed 50% of the mean value where selected. Genes significantly affected by exogenous Suc were identified by comparing the datasets by using the Microarray Analysis Suite 5.0 software algorithm. Only genes showing a significant change in both the biological replicates were selected (see Fig. 5A legend for details). Clustering was performed using the MAPMAN software (Thimm et al., 2004).

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