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Original Article

A reassessment of the role of sucrose synthase in the hypoxic sucrose-ethanol transition in Arabidopsis

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ABSTRACT

Plants under low-oxygen availability adapt their metabolism to compensate for the lower ATP production that arises from the limited respiratory activity in mitochondria. Anaerobic glycolysis requires continuous fuelling of carbon units, also provided from sucrose. The anaerobic catabolism of sucrose is thought to require the activity of sucrose synthase, being this enzymatic reaction more energetically favourable than that of invertase. The role of sucrose synthases (SUS) for aerobic sucrose catabolism in Arabidopsis has been recently questioned since *SUS* mutants fail to show altered phenotype or metabolic profile. In the present paper, we analysed the role of SUS1 and SUS4, both induced by low oxygen, in plant survival and ethanol production. The results showed that mutants lacking both SUS were as tolerant to low oxygen as the wild type in most of the experimental conditions tested. Only under conditions of limiting sugar availability the requirement of SUS1 and SUS4 for ethanol production was evident, although partly compensated by invertase activities, as revealed by the use of a double mutant lacking the two major cytosolic invertases. We conclude that, contrary to general belief, the sucrose synthase pathway is not the preferential route for sucrose metabolism under hypoxia.

Key-words: *Arabidopsis thaliana*; anoxia; hypoxia; invertase; submergence.

INTRODUCTION

Sucrose represents the major translocated sugar from photosynthetic source tissues to sink organs, where subsequent cleavage to its component hexoses represents the starting step for utilization of the photoassimilates in various metabolic processes. In plants, there are only two enzymatic groups that are able to catalyse sucrose degradation, namely sucrose synthases (SUS) and invertases. SUS catalyses the reversible conversion of sucrose and uridine diphosphate (UDP) into UDP-glucose and fructose. UDP-Glc may then be used for cellulose synthesis (Ruan *et al.* 1997) or converted to Glc-1-P, via an inorganic pyrophosphate (PPi)-dependent reaction, catalysed by UDP-Glc pyrophosphorylase (UGPase). Hexose phosphates resulting from this reaction are then used in glycolysis or other metabolic processes. Invertase catalyses the irreversible hydrolysis reaction of sucrose into fructose and glucose, which are subsequently phosphorylated by hexo- and fructokinases (Renz *et al.* 1993), using ATP or uridine triphosphate (UTP) as energy donors. The two sucrose-degrading pathways differ in their overall energy costs related to fuelling the hexoses resulting from sucrose hydrolysis to glycolysis. Sucrose degraded via invertase requires two molecules of ATP to be converted into hexose-phosphates, while the SUS pathway linked to UGPase requires only one molecule of PPi (Huber & Akazawa 1986; Stitt 1998).

The reaction catalysed by SUS, requiring less energy, should be preferred under conditions of energy limitation. Indeed, some *SUS* genes are induced in response to low oxygen in almost all plants species tested, including rice (Richard *et al.* 1991; Guglielminetti *et al.* 1995), maize (Springer *et al.* 1986), wheat (Albrecht *et al.* 1993; Albrecht & Mustroph 2003), potato (Biemelt *et al.* 1999; Bologa *et al.* 2003) and Arabidopsis (Baud *et al.* 2004; Bieniawska *et al.* 2007). It is assumed that the importance of sucrose synthase under hypoxia is linked to its role in providing carbon units for fermentation (Guglielminetti *et al.* 1995). In corn, sucrose synthase is encoded by two genes, namely *Sh1* and *Sus1* (Guglielminetti *et al.* 1996). Only *Sh1* is induced by hypoxia, but the knockout line *sh1* produces as much ethanol as the wild type because of ectopic expression of *Sus1* in the *sh1* mutant (Guglielminetti *et al.* 1996). Ricard *et al.* (1998) showed that a double-mutant *sh1*/*sus1* displays reduced ethanol production and survival to hypoxia, confirming the role of sucrose as the major source of carbon for ethanol synthesis. Remarkably, the invertase mRNA level and enzyme activity declines under hypoxia, providing further support for the importance of sucrose synthases over invertases in driving sucrose catabolism under low-oxygen conditions in cereals (Bertani *et al.* 1981; Guglielminetti *et al.* 1995, 1996; Perata *et al.* 1997; Ricard *et al.* 1998; Bologa *et al.* 2003). In dicots the picture is less clear. Biemelt *et al.* (1999) showed that reduced sucrose synthase activity in transgenic potato plants has a negative impact of survival but did not restrict glycolysis and ATP level, as would have been expected if sucrose synthase was required to fuel glycolysis and fermentation under hypoxia. Bologa *et al.* (2003) reached a different conclusion, suggesting that the sucrose synthase pathway is metabolically

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advantageous in potato under hypoxia. More recently, Bieniawska *et al*. (2007) analysed the *SUS* family in Arabidopsis, which is composed of six genes (*SUS1* to *SUS6*). Only *SUS1* and *SUS4* are induced under low-oxygen conditions, and a double-mutant *sus1*/*sus4* shows reduced survival after submergence, suggesting that sucrose synthase plays a role in the acclimation of Arabidopsis to hypoxia. However, all the metabolic parameters measured in the mutant in air were identical to the wild type, suggesting that invertase activities were sufficient to support sucrose metabolism under aerobic conditions, a conclusion that was strengthened by the lack of a clear phenotype in a quadruple SUS mutant (*sus1/sus2/sus3/sus4*; Barratt *et al*. 2009).

Although there is evidence of a role for sucrose synthase as part of the acclimation mechanism to anoxia in dicots (Biemelt *et al*. 1999; Bieniawska *et al*. 2007), it is still unclear if sucrose synthase is required for the sucrose to ethanol transition. In the present work, we studied an Arabidopsis *sus1*/*sus4* mutant to understand the contribution of sucrose synthases to the fermentative metabolism linked to the ability to survive low-oxygen conditions.

MATERIAL AND METHODS

Plant material and growth conditions

The *Arabidopsis thaliana* accession Columbia-0 (*Col-0*) and the mutant *pgm* (Caspar *et al*. 1985), *sus1* (*At5g20830*; SALK line N544615) and *sus4* (*At3g43190*; SALK line N619660) lines were used. *Col-0* (N1093) and *pgm* (N210) were obtained from the Nottingham Arabidopsis Stock Centre (www.arabidopsis.info). A schematic representation of the T-DNA insertions sites is shown in Supporting Information Fig. S1. The homozygous double-mutant *sus1*/*sus4* was obtained by crossing the *sus1* and *sus4* single mutants and selecting the F2. The *sus1*/*sus4* mutant in this study is thus different from the one used by Bieniawska *et al*. (2007). However, experiments performed using both the double mutants showed that they behave identically in terms of ethanol production and tolerance (data not shown). The mutations in the *SUS* genes were confirmed by PCR using the following primer couples: SUS1-F AACTTGTATGG ATTCCTAAACACCA + SUS1-R GTTTAATGTTGAGT CCTTGTTGCTT; LBb1 AACCAGCGTGGACCGCTTG CTG + SUS1-R GTTTAATGTTGAGTCCTTGTTGCTT; SUS4-F CTTTTCTCAAAATTTGCAATAGCTC + SUS4-R TTAATTTCCTCTTACCACCAATCAA; LBb1AACC AGCGTGGACCGCTTGCTG + SUS4-F CTTTTCTCAA AATTTGCAATAGCTC. The invertase mutants *cinv1*, *cinv2* and *cinv1/cinv2* were kindly provided by Dr. Alison M. Smith (John Innes Centre, Norwich, UK). Seedlings used for the anoxia, hypoxia and submergence experiments were produced from seeds sterilized with diluted bleach (10 min incubation in 1.7% v/v sodium hypochlorite, rinsed and washed seven times in sterile water). Seedling were grown in six-well plates containing 2.5 mL of liquid Murashige and Skoog (MS) half-strength solution. The multi-well plates were incubated in the dark at 4 °C for 2 d (vernalization, to promote homogeneous germination) and then transferred to 23 °C for 4 d in the dark before the treatments. Treatments under anoxia and hypoxia (Fig. 3a,b) were carried out using seedlings grown on vertical plates containing MS half-strength solution, 1% agar and 30 mM sucrose. Submergence treatments (Fig. 3c) were performed using seedlings grown in small glass jars containing MS half-strength solution, 1% agar and 30 mM sucrose. The phenotype of treated plants was evaluated using 2 weeks-old seedlings grown under a 12/12 h photoperiod at 150 μmol photons m^{-2} s^{-1}. The low-oxygen treatments were performed in the dark. The plates (Fig. 3a,b) were then transferred back to a 12/12 h photoperiod at 150 μmol photons m^{-2} s^{-1} for a 1-week-long recovery phase. The plants subjected to submergence (Fig. 3c) were photographed at the end of the treatment (without recovery phase). Survival experiments were repeated three times using 30 plants per genotype in each replication. Anoxic treatments were carried out in the dark as previously described (Banti *et al*. 2008). Hypoxia was obtained using a chamber with a continuous flux of gas at 1% O_2. Submergence of seedlings grown in glass jars was obtained filling the jar with sterile water. Waterlogging of adult plants was performed using 3-week-old plants grown in a hydroponic system (Mithran *et al*. 2014). Plants were waterlogged up to the petiole with the nutrient solution and kept under the 12/12 h photoperiod at 150 μmol photons m^{-2} s^{-1}. Plants were photographed after a week of recovery from the submergence treatment. The leaf area was measured using ImageJ (http://imagej.nih.gov/ij/). The treatments are summarized in Supporting Information Table S1.

Gene transcript abundance analysis

Total RNA was extracted as previously described (Perata *et al*. 1997), with minor modifications (omission of aurintricarboxylic acid) to make the protocol compatible with the subsequent PCR procedures. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check for RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion, www.ambion.com). RNA was then reverse-transcribed using an iScript™ cDNA synthesis kit (BioRad Laboratories, Hercules, CA, USA). Expression analysis was performed by real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using 40 ng cDNA and iQ™ SYBR® Green Supermix (Biorad Laboratories), according to the manufacturer's instructions. Expression of Ubiquitin10 (*At4g05320*) was used as an endogenous control. Relative expression levels were calculated using GeNorm (http://medgen.ugent.be/~jvdesomp/genorm). The primers used for *SUS* transcripts were as described by Bieniawska *et al*. (2007), Licausi *et al*. (2010) (ADH) and Mithran *et al*. (2014) (Ubiquitin10). The expression of sucrose synthase and invertase genes was also analysed using the publicly available microarray datasets using Genevestigator (Hruz *et al*. 2008). Invertase and sucrose synthase activities were measured as previously reported (Guglielminetti *et al*. 1995).

Ethanol assay

Ethanol released in the external liquid medium was measured, with the exception of plants grown in pots that were assayed for their ethanol content in the root tissues. For tissues ethanol extraction, frozen samples were powdered with liquid nitrogen and homogenized with 200 μL ice-cold 0.6 mM $HClO_4$. After standing on ice for 30 min with occasional shaking, the homogenate was centrifuged at 30 000 *g* for 15 min at 4 °C. The supernatant was centrifuged (20 000 *g*, 5 min) after neutralization with 3 M KOH, and the resulting supernatant was frozen in liquid nitrogen and kept at –80 °C until assayed (Bouny & Saglio 1996). Samples were assayed through coupled enzymatic assay methods, measuring the increase in A_{340}, as described by Banti *et al.* (2008).

ATP/ADP assay

ATP and ADP were extracted from seedlings tissue (about 100 mg fresh weight) using trichloroacetic acid (TCA) as previously described by Herbers *et al.* (1997). The samples were ground in liquid nitrogen, 1 mL of 16% (w/v) TCA in diethylether (4 °C) was added, further mixed and kept on ice for 20 min. 0.6 mL of 16% TCA (w/v) in water containing 5 mM EGTA (4 °C) was added to the homogenate. The samples were left at least 1 h (up to 3 h) at 4 °C. After centrifuging for 10 min (18 000 *g*), the water phase was extracted three times with water-saturated with ether, and then neutralized with 5 M KOH. ATP and ADP were measured by high-performance liquid chromatography (HPLC) with SHIMADZU C18 column. The data reported in Figs 4 and 5 were obtained using the ATP assay kit ENLITEN® Luciferase/Luciferin reagent (Promega, Madison, WI, USA), according to the manufacturer specifications.

RESULTS

SUS genes are differentially expressed in Arabidopsis seedlings under low-oxygen conditions

The sucrose synthase gene family in Arabidopsis is composed of six member, two of which, *SUS1* and *SUS4*, are induced by low-oxygen conditions (Baud *et al.* 2004; Supporting Information Fig. S2). In maize, the *sh1* mutation results in the ectopic expression of the SUS1-encoded isoform and this prompted us to verify the expression of all six Arabidopsis *SUS* genes in the wild type (Col-0) as well as in the single *sus1* and *sus4* mutants and the double-mutant *sus1*/*sus4*. Three distinct low-oxygen conditions were used, including anoxia, hypoxia (1% oxygen) and submergence. The results showed that all treatments were effective in inducing the alcohol

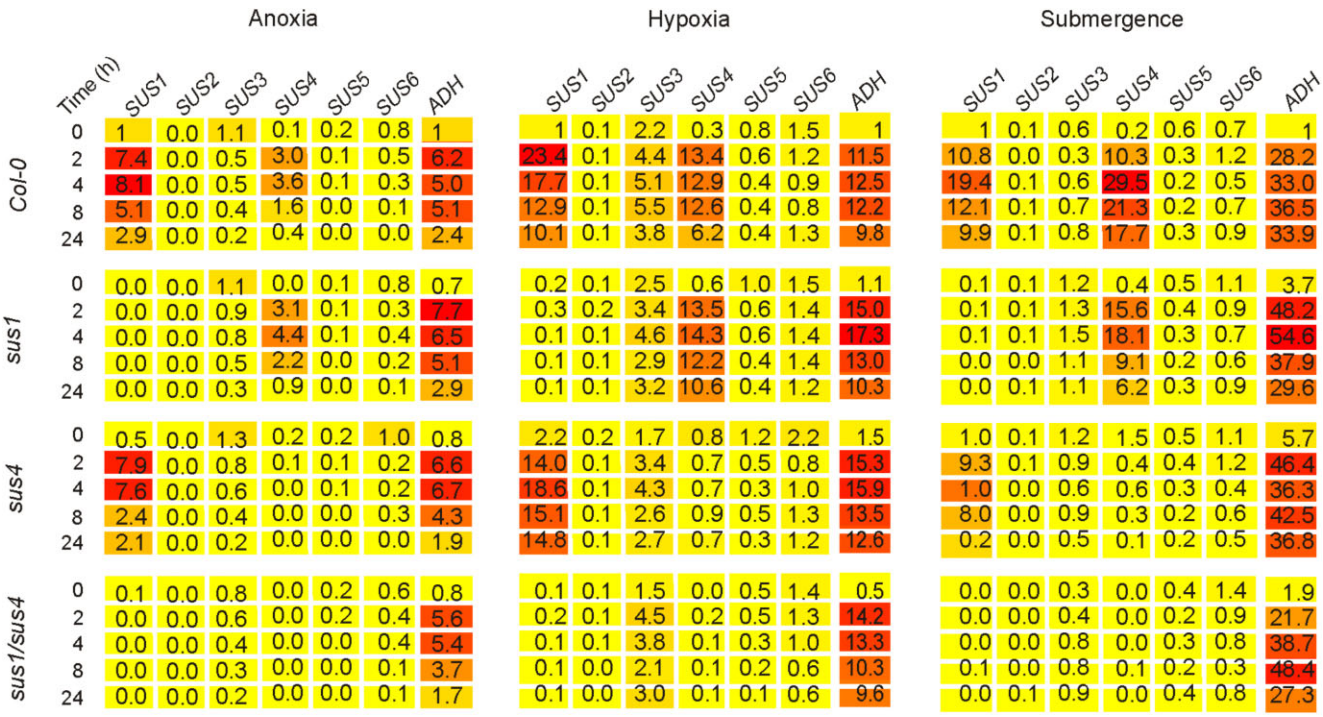

Figure 1. Expression pattern of sucrose synthase genes under low-oxygen conditions. Arabidopsis seedlings were grown and treated under low-oxygen conditions as described in the Material and Methods section. Four-day-old seedlings (dark grown) were grown in multi-well plates with liquid media containing sucrose (90 mM). Anoxia, hypoxia and submergence lasted 48 h in the darkness. See Supporting Information Table S1 for details about the experimental conditions. The change in expression due to low-oxygen conditions is reported as fold change versus the aerobic control, calculated setting to 1 the value of *SUS1* at time 0 h. Data are shown as heat maps, with high levels of expression shown in red and low levels of expression in yellow. Data are mean of three technical replicates from a representative experiment. ±SD did not exceed 10% of the mean values.

dehydrogenase (*ADH*) gene, thus indicating that the level of oxygen in all treatments dropped below the threshold for the induction of anaerobic genes (Fig. 1). Under anoxia, the induction of *SUS1* was stronger than that of *SUS4*. A faint PCR signal was detected for *SUS1* in the *sus1* mutant (*Ct* value >31), but this is most likely a consequence of a minor cross-reaction of the primers used with other SUS transcripts. The *sus1* mutant is likely a knockout for this gene, being induction by low oxygen abolished in the mutant. None of the other five genes displayed an altered pattern of expression as a consequence of the lack of *SUS1*. The same was true for the *sus4* mutant and the double *sus1/sus4* mutant was clearly

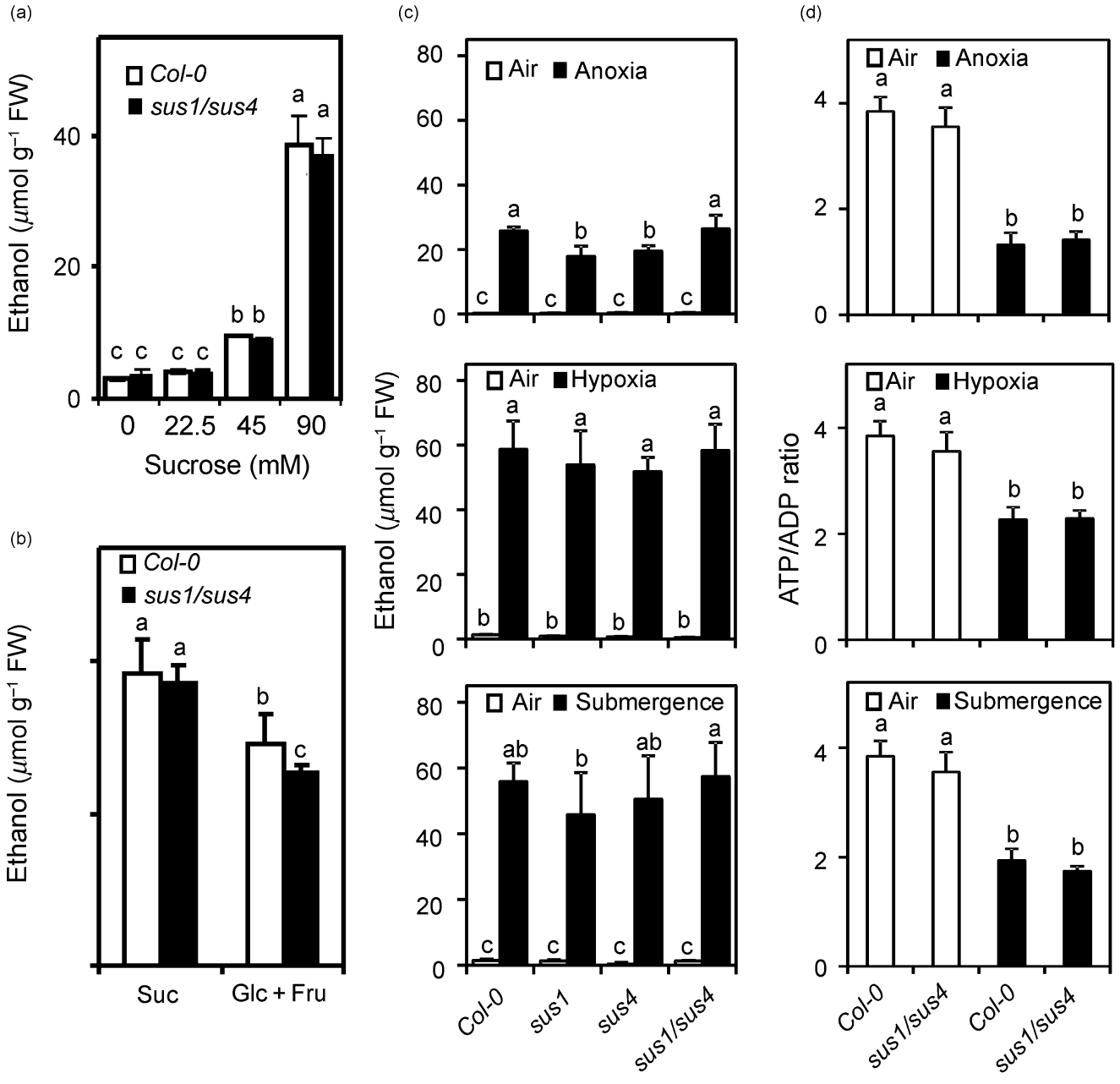

Figure 2. Impact of *SUS* mutations on ethanol production and ATP/ADP ratio under low-oxygen conditions. Arabidopsis seedlings were grown and treated under low-oxygen conditions as described in the Material and Methods section. See Supporting Information Table S1 for details about the experimental conditions. (a) Effect of exogenous sucrose on ethanol production. Four-day-old seedlings (dark grown) were grown in multi-well plates with liquid media containing sucrose (0–22.5–45–90 mM). Anoxia lasted 48 h in the darkness. (b) Effect of exogenous sucrose (90 mM) or glucose (90 mM) + fructose (90 mM) on ethanol production. Anoxia lasted 48 h in the darkness. (c) Ethanol production in the presence of 90 mM sucrose under anoxia, hypoxia, submergence. (d) ATP/ADP ratio in the presence of 90 mM sucrose under anoxia, hypoxia, submergence. Data (±SD) were subjected to one-way and two-way analysis of variance (ANOVA; P-value ≤ 0.05) and the means were separated using the F-test (95.0% confidence level). See Supporting Information Table S2 for details about the statistical analysis.

unable to induce any of the *SUS* genes (Fig. 1). When analysing the expression of *SUS* genes under conditions less harsh than anoxia (hypoxia and submergence), we noticed a higher increase of the expression of *SUS4* over anoxia and, limited to hypoxia, a weak expression of *SUS3* (Fig. 1). Overall, the induction of *SUS1* and *SUS4* is clearly present under all the anaerobic growth conditions used. Besides *SUS1* and *SUS4*, none of the other *SUS* genes, with the only exception of the low level of *SUS3* transcript, showed elevation by hypoxia. The use of the *sus1*/*sus4* mutant thus represents a valid tool to investigate the importance of SUS under low-oxygen conditions.

SUS1 and *SUS4* are not required for the sucrose-ethanol transition in seedlings under low-oxygen conditions

The availability of the double (*sus1/sus4*) allowed us to investigate the impact of this mutations on ethanol production under low-oxygen conditions. Ethanol production in wild-type Arabidopsis seedlings was enhanced by 90 mM sucrose versus control or lower sucrose concentrations (Fig. 2a), with no differences observed in the amount of ethanol produced by the *sus1*/*sus4* mutant when compared with the wild type (Fig. 2a). Sucrose represents a better carbon source when compared with a 1:1 mixture of glucose and fructose (Fig. 2b). None of the sucrose synthase mutants displayed lower ethanol production under either anoxia, hypoxia and submergence (Fig. 2c). Energy production under low-oxygen conditions relies on the use of sugars through the fermentative metabolism. Given that sucrose was clearly enhancing ethanol production (Fig. 2a), a lower ATP production would be expected when the energy-advantageous sucrose synthase pathway was inactive as a consequence of mutations in the *SUS1* and *SUS4* genes. When comparing the consequences of mutations in the *SUS1* and *SUS4* genes in terms of fermentation, we noticed that, surprisingly, the amount of ethanol produced was the same in all genotypes (Fig. 2a,b,c). Even the *sus1*/*sus4* mutant produced exactly the same amount of ethanol of the wild type under all the experimental conditions used. The level of ATP decreased as a consequence of the low-oxygen treatments, with anoxia leading to the lowest ATP/ADP ratio (Fig. 2d). The lack of differences in ethanol production would predict that also the amount of ATP produced by the *sus1*/*sus4* mutant was not altered when compared with the wild type. We indeed found that the ATP/ADP ratio was the same in the wild type and *sus1*/*sus4* mutant (Fig. 2d).

Mutants defective in sucrose synthase display a conditional phenotype in terms of low-oxygen tolerance

Tolerance to hypoxia is lower in corn (Ricard *et al*. 1998) and potato (Biemelt *et al*. 1999) with reduced sucrose synthase activity. We tested whether the absence of *SUS1* and *SUS4* negatively impacted survival to low oxygen. In our experimental conditions, plants rapidly died after 22 h of anoxia, but no differences were observed when comparing the *sus* mutants with the wild type (Fig. 3a). Under hypoxia, all genotypes survived well up to 7 d (Fig. 3b), while submerged seedlings started displaying intolerance symptoms after 14 d (Fig. 3c), with no differences among genotypes. These results apparently contrast with the conclusions reached by Bieniawska *et al*. (2007), who showed that pot-grown *sus1/sus4* mutant was less tolerant to waterlogging. We therefore performed a waterlogging experiment by using the same conditions described by Bieniawska *et al*. (2007). The results confirmed a lower tolerance of the *sus1*/*sus4* mutant (Fig. 4a). The *sus1*/*sus4* mutant showed reduced recovery after 4 d waterlogging, with most of the leaves showing senescence symptoms (Fig. 4a,b). This result indicated that the role of SUS under low oxygen may be conditional, apparently occurring in adult plants subjected to waterlogging. The question arising is if under these conditions, the production of ethanol is affected by the lack of *SUS1* and *SUS4*. We therefore grew Arabidopsis plants using a hydroponic system that allows easy harvest of roots, and confirmed that the *sus1*/*sus4* mutant displays intolerance symptoms when waterlogged

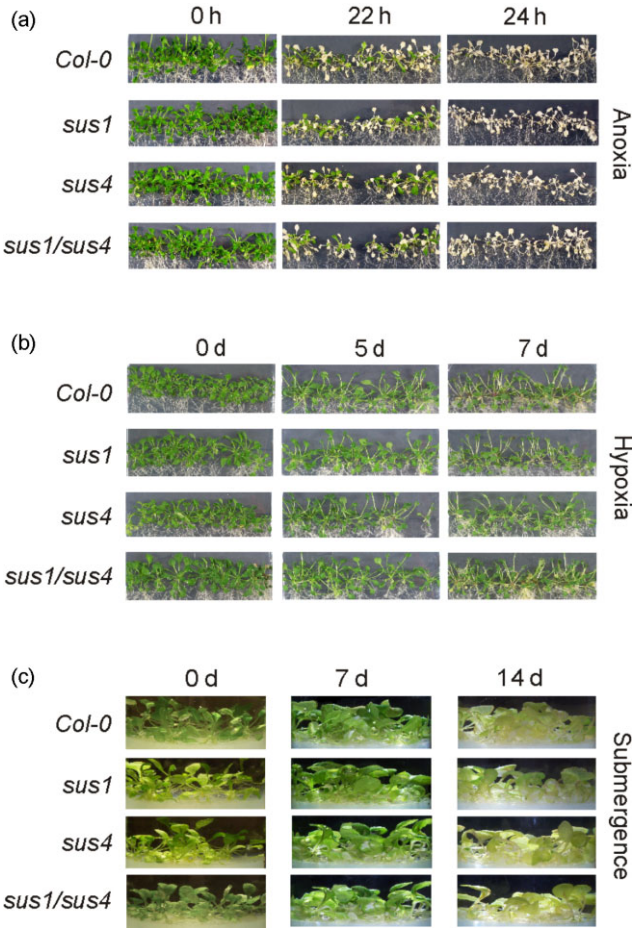

Figure 3. Arabidopsis under low-oxygen conditions. Arabidopsis seedlings were grown and treated under low-oxygen conditions. (a) anoxia (vertical plates, 1% agar, sucrose 30 mM), (b) hypoxia (vertical plates, 1% agar, sucrose 30 mM), (c) submergence (glass jars, 1% agar, sucrose 30 mM). See Supporting Information Table S1 for details about the experimental conditions.

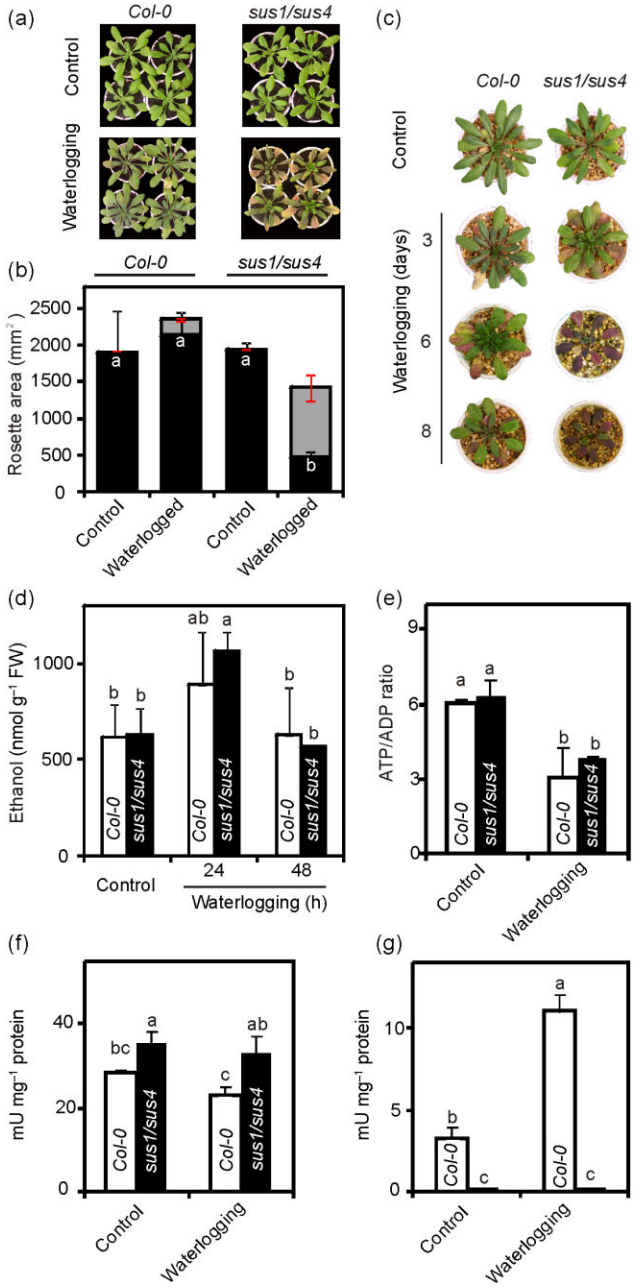

Figure 4. Tolerance to waterlogging and ethanol production in Arabidopsis plants grown in pots. (a) Arabidopsis plants were grown in pots with soil. See Supporting Information Table S1 for details about the experimental conditions. Plants were submerged up to the leaf petioles (waterlogging) for 4 d. Photographs were taken 1 week after the end of the submergence treatment. (b) Rosette area of plants treated as shown in panel a. The data are mean from 10 biological replicates (±SD). The black bars represent the area of green, vital leaves, while the grey bar represent senescent, purplish leaves. (c) Arabidopsis plants were grown in pots using a hydroponic system with nutrient solution, as described in the Material and Methods section. See Supporting Information Table S1 for details about the experimental conditions. Plants were submerged up to the leaf petioles (waterlogging). Photographs were taken 1 week after the end of the submergence treatment. The experiment was repeated three times with consistent results. All plants were very similar to those shown in the photographs. (d) Ethanol production in roots of the plants treated as described above for 24 and 48 h. (e) ATP/ADP ratio in aerobic (control) roots and waterlogged roots (48 h). (f) Invertase activity in aerobic (control) roots and waterlogged roots (48 h). (g) Sucrose synthase activity in aerobic (control) roots and waterlogged roots (48 h). Data (±SD) were subjected to one-way and two-way analysis of variance (ANOVA; P-value ≤ 0.05) and the means were separated using the F-test (95.0% confidence level). See Supporting Information Table S2 for details about the statistical analysis.

also under these experimental conditions (Fig. 4c). A limited amount of ethanol was detected in roots of Arabidopsis plants, with a modest increase as a consequence of the waterlogging treatment (Fig. 4d). Although most of the ethanol has probably diffused out of the roots, there was however no difference in ethanol production when comparing the wild type with the *sus1*/*sus4* mutant, again suggesting that sucrose synthase does not influence ethanol production (Fig. 4d). Consistently, although waterlogging reduced the ATP/ADP ratio, we did not detect differences between the wild type and the *sus1/sus4* mutant (Fig. 4e). Sucrose synthase activity is low in Arabidopsis seedlings, often below the detection limit (data not shown), but it was readily measurable in root extracts from adult plants. Invertase activity is slightly higher in the *sus1/sus4* mutant, with negligible effects of waterlogging (Fig. 4f). Sucrose synthase activity was clearly enhanced by waterlogging but was almost undetectable in roots of the *sus1/sus4* mutant (Fig. 4g), indicating that SUS1 and SUS4 represent the major isoforms in Arabidopsis roots.

Both sucrose synthase and invertases contribute to ethanol production under anoxia

A recent paper showed that a quadruple *sus1/sus2/sus3/sus4* mutant is devoid of sucrose synthase in all cell types except the phloem (where *SUS5* and *SUS6* are expressed) but displays a normal phenotype and no alterations in sugar metabolism (Barratt *et al.* 2009). A double mutant for cytosolic invertase 1 and 2 (*cinv1/cinv2*) showed instead a very stunted growth, leading to the conclusion that normal growth in Arabidopsis requires cytosolic invertase but not sucrose synthase (Barratt *et al.* 2009). None of the nine invertase transcripts is affected by low oxygen (Supporting Information Fig. S3). We compared the production of ethanol under anoxia by comparing the *sus1*/*sus4* mutant with the *cin1*, *cin2* and *cinv1*/*cinv2* mutants. Growth of the *cinv1/cinv2* mutant requires exogenous glucose (Barratt *et al.* 2009). We therefore grew the seedlings in the presence of both glucose and sucrose to minimize differences in the metabolic status of the different genotypes before the treatments. Different from the experiment described in Fig. 2 where seedlings were placed under low-oxygen in the presence of exogenous sucrose, we replaced the sucrose/glucose-containing medium with mannitol immediately before the beginning of the anoxic treatment. The results showed reduced ethanol production in the *sus1/sus4* mutant during the first 12 h under anoxia (Fig. 5a) and a significant reduction of ethanol

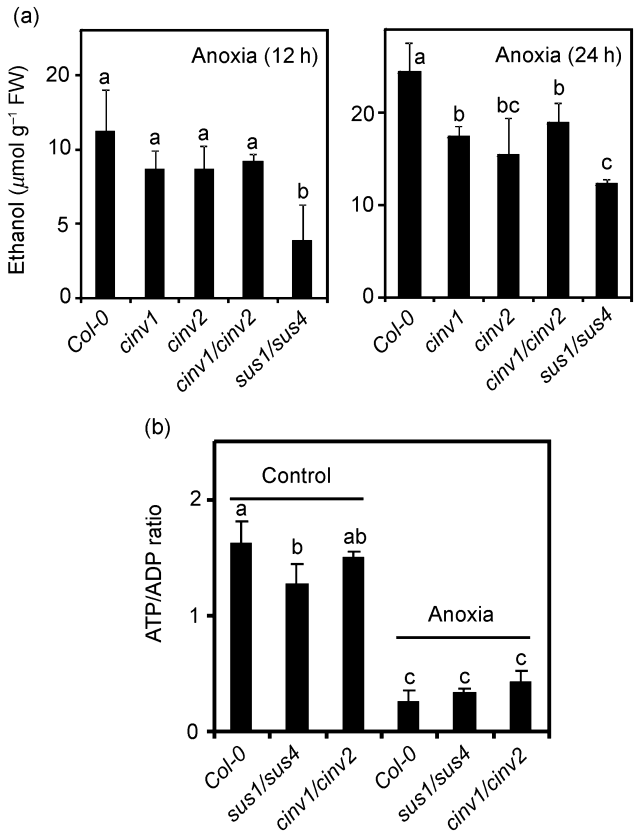

Figure 5. Effect of mutations in the *SUS* and invertase genes on ethanol production. Arabidopsis seedlings were grown and treated under anaerobic conditions as described in the Material and Methods section. See Supporting Information Table S1 for details about the experimental conditions. (a) Ethanol production in seedlings that were grown in the presence exogenous glucose (55 mM) and sucrose (15 mM). Treatment under anoxia was performed after removing the sugar-containing medium, replaced with mannitol (70 mM). (b) ATP/ADP ratio in aerobic (control) and anoxic seedlings treated as in panel a. Data (±SD) were subjected to one-way and two-way analysis of variance (ANOVA; P-value ≤ 0.05) and the means were separated using the F-test (95.0% confidence level). See Supporting Information Table S2 for details about the statistical analysis.

production also in the invertase mutants after 24 h of anaerobiosis (Fig. 5a), highlighting a contribution of both sucrose synthases and invertases in ethanol production under anoxia. The ATP/ADP ratio was, however, almost identical when comparing the wild type with the *sus1/sus4* and the *cinv1/cinv2* mutants (Fig. 5b).

DISCUSSION

Sucrose metabolism requires the action of either sucrose synthases or invertases. Sucrose synthase has been considered to represent an essential enzyme for the entry of sucrose in the metabolism, also because the reaction catalysed by SUS is energetically less costly than the one driven by invertases (Bailey-Serres & Voesenek 2008). Recently, the importance of sucrose synthases in plant sugar metabolism has been questioned. Mutants devoid of sucrose synthase genes were shown to have unaltered sugar metabolism and phenotype (Bieniawska *et al.* 2007). In the work by Barratt *et al.* (2009) a quadruple *SUS* mutant (*sus1/sus2/sus3/sus4)* with undetectable SUS activity displayed a wild-type phenotype and was also normal in terms of starch, sugar, cellulose content as well as cell wall structure. In contrast, a double mutant missing two out of the nine neutral invertase isoforms was severely impaired in growth (Barratt *et al.* 2009). How do these results relate with the evidences of a crucial role of sucrose in some plant organs? Barratt *et al.* (2009) proposed that sucrose synthase might be required under specific environmental conditions, such as those imposing limited oxygen availability. It has been indeed shown that reduced sucrose synthase activities result in specific phenotypes mostly in bulky organs such as fruits, seeds and tubers, which are known to have a limited oxygen content (Geigenberger 2003). Remarkably, a *sus1*/*sus4* mutant that did not show any clear phenotype or metabolic alteration was shown to possess limited tolerance to waterlogging, thus supporting the view of a conditional role of SUS under specific environmental conditions (Bieniawska *et al.* 2007). It would be clear to assume that the role of the SUS1 and SUS4 isoforms, which are induced under low-oxygen conditions (Baud *et al.* 2004), is to channel sucrose into the ethanol fermentation. Our results indicate that there is no strict requirement for the induction of *SUS1* and *SUS4* to support the conversion of sucrose to ethanol. Sucrose is indeed the best substrate for ethanol production (Fig. 2b). Contrary to expectation, the double *sus1*/*sus4* mutant produced as much ethanol as the wild type (Fig. 2a,b,c) and also the ATP content, which is reduced by low oxygen, was not different in the wild type versus the *sus1*/*sus4* mutant (Fig. 2d). Tolerance to low oxygen in the *SUS* mutants was not reduced, except for adult plants grown in pots (Fig. 4a,b,c), confirming the results of Bieniawska *et al.* (2007). However, the production of ethanol under these conditions was very limited and not different from the wild type in the *sus1*/*sus4* mutant (Fig. 4d), and also the ATP level was the same in the wild type and the *sus1*/*sus4* mutant (Fig. 4e). A lack of correlation between tolerance and metabolism was also observed by Biemelt *et al.* (1999) in potato. The evidence of a major role of invertase for Arabidopsis growth prompted us to analyse the consequence of the *cinv1*/*cinv2* mutation on ethanol production. The results highlighted a reduction in ethanol production as a consequence of either *sus1*/*sus4* or *cinv1*/*cinv2* mutations, but only when the amount of soluble sugars available was limiting (Fig. 5a). This suggests that seedlings that are provided with large exogenous sucrose availability can bypass the SUS1/SUS4-dependent step for sucrose metabolism, indicating a possible role for apoplastic invertase under such non-physiological experimental conditions. In corn, the expression of two invertases was repressed under hypoxia, concomitantly with increased sucrose synthase expression (Zeng *et al.* 1999) and there is no evidence of up-regulation of invertase genes by low oxygen in Arabidopsis (Supporting Information Fig. S3). It was thus unexpected to observe an

impact of the *cinv1*/*cinv2* mutations on ethanol production, to an extent not far from that of the *sus1*/*sus4* mutations (Fig. 5a). However, the invertase activity detected in Arabidopsis roots was largely exceeding that of SUS, even under waterlogging conditions, thus indicating that invertases can easily compensate for the lack of SUS in the *sus1/sus4* mutant (Fig. 4f,g). This suggests that the induction of *SUS1* and *SUS4* under low oxygen has an impact on the anaerobic metabolism of Arabidopsis, but this does not represent a strict requirement for providing carbon units deriving from sucrose to fuel the production of ethanol. Given the relative contribution of sucrose synthases over invertases in the fermentative metabolism, the energetic advantage that is inherent to the sucrose synthase pathway is questionable (Fig. 5b) and raises questions about the reasons behind the reduced tolerance to submergence of the *sus1/sus4* mutant observed by Bieniawska *et al*. (2007) and ourselves (Fig. 4a,b,c). Biemelt *et al*. (1999) suggested that the major role of sucrose synthase in potato under hypoxic conditions is not to fuel glycolysis but may instead play a role in channelling carbon into cell wall polymers. Changes in cell wall composition are observed in rice, wheat and *Rumex* plants subjected to submergence (Azuma *et al*. 1996; Albrecht & Mustroph 2003; Groeneveld & Voesenek 2003), but genes involved in cell wall synthesis are known to be repressed by submergence in rice, Arabidopsis and *Rorippa* plants (Sasidharan *et al*. 2013 and reference therein). In Arabidopsis grown under aerobic conditions, sucrose synthases do not contribute to cellulose synthesis (Bieniawska *et al*. 2007; Barratt *et al*. 2009) and it is unclear at present why providing UDP-glucose for cellulose synthesis via sucrose synthase would be a requirement for tolerance to submergence. Furthermore, we cannot rule out the possibility that sucrose synthase contributes to support sucrose metabolism during the recovery from waterlogging. These aspects of sucrose synthase function under hypoxia certainly deserve further study.

The evidence showing that the invertase pathway is largely predominant for sucrose metabolism linked to plant growth in Arabidopsis (Barratt *et al*. 2009) opens the discussion about the function of the sucrose synthase in plant metabolism. The strong anaerobic induction of *SUS1* and *SUS4* (Supporting Information Fig. S2), together with the stunted phenotype of the *sus1*/*sus4* mutant grown under waterlogging (Bieniawska *et al*. 2007; Fig. 4a,b,c), suggested that the role of SUS1 and SUS4 is to fuel the anaerobic metabolism at specific developmental stages or metabolic states (Barratt *et al*. 2009), but the results reported here indicate that, although these isoforms do indeed play a role in providing carbon units for ethanol production under low-oxygen conditions, this does not have a major influence on ethanol production. Invertases appear to be able to largely compensate for the loss of both SUS1 and SUS4 and indicate that, contrary to general belief, the sucrose synthase pathway is not the preferential route for sucrose metabolism under hypoxia. Instead, both SUS and invertases contribute to sucrose metabolism under anaerobic conditions.

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SUPPORTING INFORMATION

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

Figure S1. Schematic representation of the T-DNA insertion in the mutants for *SUS1* (N544615) and *SUS4* (N619660). The promoter region is represented in blue, the UTR regions in grey, the exons in black. LB indicates the left border of the T-DNA sequence.

Figure S2. Microarray analysis of the six sucrose synthase genes using the Genevestigator Tool. Datasets related to low-oxygen treatments were selected.

Figure S3. Microarray analysis of the nine invertase genes using the Genevestigator Tool. Datasets related to low-oxygen treatments were selected.

Table S1. Growth media, conditions, treatments used for the experiments reported in the figures.

Table S2. Data were subjected to one-way and two-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95.0% confidence level). Significance of two-way ANOVA (**P*-value ≤ 0.05; ****P*-value ≤ 0.001; n.s. = not significant).