Steady sucrose degradation is a prerequisite for tolerance to root hypoxia

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We investigated the role of glycolysis and sucrolysis in the difference in tolerance to root hypoxia between two Myrtaceae tree species, Melaleuca cajuputi (which shows superior tolerance to root hypoxia) and Eucalyptus camaldulensis (which does not). Analysis of the adenylate energy charge (AEC) in roots subjected to a 4-day hypoxic treatment (HT) in hydroponic culture revealed that the interspecies difference in tolerance corresponds to the ability to maintain energy status under root hypoxia: AEC was reduced by HT in E. camaldulensis, but not in M. cajuputi. The energy status in HT roots of E. camaldulensis was restored by feeding of glucose (Glc) but not sucrose (Suc). These data provide evidence that low substrate availability for glycolysis resulting from an impairment of sucrolysis suppresses ATP production under hypoxic conditions in this species. Measurements of the rates of O2 consumption and CO2 production in roots indicated that E. camaldulensis, but not M. cajuputi, failed to activate fermentation in HT roots. These results cannot be attributed to enzymatic dysfunction, because no inhibition of main glycolytic and fermentative enzymes was observed in both species, and Glc feeding had a beneficial effect on AEC of HT roots of E. camaldulensis. The impairment of sucrolysis was demonstrated by inhibited soluble acid invertase activity in HT roots of E. camaldulensis. In contrast, there was no inhibition in all sucrolytic enzymes tested in HT roots of M. cajuputi, suggesting that steady Suc degradation is essential for maintaining high energy status under root hypoxia. We conclude that root sucrolysis is one of the essential factors that determines the extent of tolerance to root hypoxia.

Keywords: flooding, glycolytic flux, invertase, oxygen deficiency, sucrose synthase.

Introduction

Reforestation is urgently needed to restore biomass production on degraded lands. Degraded lands, including wetlands such as swamps and marshes, where flooding impairs the biomass productivity of planted trees, exist in various locations worldwide (Pezeshki 2001, Effler and Goyer 2006). Reforestation of degraded wetlands requires flood-tolerant tree species. Soil flooding results in root hypoxia because of the low diffusion rate of gases in water and therefore low O2 supply (Armstrong 1979). Since O2 deficiency leads to reduced ATP production by oxidative phosphorylation in mitochondria, root hypoxia is likely to cause energy deficiency in plant roots (Ricard et al. 1994, Vartapetian and Jackson 1997). Flood-tolerant tree species are assumed to possess metabolic mechanisms to prevent energy deficiency during root hypoxia (Pezeshki 2001).

One possible mechanism for maintaining the energy status under hypoxia is the activation of fermentation (Tadege et al. 1999, Ismond et al. 2003), which involves induction of the activity of enzymes such as alcohol dehydrogenase (ADH; EC 1.1.1.1) and pyruvate decarboxylase (EC 4.1.1.1). Previously, we compared the responses to root hypoxia (by using hydroponic culture with N2 bubbling) in two Myrtaceae tree species, Melaleuca cajuputi Powell and Eucalyptus camaldulensis Dehnh. (Kogawara et al. 2006). We found that the growth of E. camaldulensis, but not
M. cajuputi, was inhibited, although ADH activity was induced similarly under hypoxia in the roots of both species (Kogawara et al. 2006). We speculated that factors other than activation of fermentative enzymes determine the difference in root hypoxia tolerance in these species.

For plants to take advantage of hypoxia-induced activation of fermentative enzymes, glycolytic flux must be sustained or enhanced to keep up with the demand for substrate (Bouny and Saglio 1996), which may be achieved by an increase in the activities of glycolytic enzymes (Rivoal et al. 1989, Fox et al. 1995, Bouny and Saglio 1996). In maize roots, hypoxic treatment (HT) increases hexokinase activity, which improves the energy status and survival rates of root tips under anoxia (Bouny and Saglio 1996). Using 13C-labeling, we demonstrated that HT increased the incorporation of photoassimilates into the water-soluble carbohydrate fraction of M. cajuputi roots, but did not change the total carbon pool size of this fraction, which indicates an increased carbohydrate turnover in the respiratory metabolism (Kogawara et al. 2006). Therefore, the high tolerance of M. cajuputi to root hypoxia may depend on an enhanced glycolytic flux into fermentation.

In plant roots, glycolysis substrates, namely glucose (Glc), fructose and UDP-Glc, are supplied mainly via sucrose (Suc) degradation, in which invertase (INV; EC 3.2.1.26) and sucrose synthase (SuSy; EC 2.4.1.13) play a pivotal role. Both INV and SuSy have several isoforms with different subcellular locations, which together regulate Suc partitioning between respiration, biosynthesis of structural carbohydrates and carbohydrate storage (Sturm and Tang 1999). Anoxic or hypoxic stress influences Suc degradation through inhibition of INV activity and promotion of SuSy activity, and stress sensitivity differs between isoforms (Germain et al. 1997, Zeng et al. 1999, Albrecht and Mustroph 2003, Gharbi et al. 2007); anoxia also affects the location of a SuSy isoform in maize roots (Subbaiah and Sachs 2001). These reports suggest that hypoxic or anoxic stress may alter substrate availability for glycolysis. Whether the stability of substrate supply for glycolysis by INV and SuSy isoforms under root hypoxia is associated with interspecies differences in hypoxia tolerance remains to be established.

Previously, we found that Suc accumulated in hypoxic roots of E. camaldulensis, whereas hexoses but not Suc accumulated in M. cajuputi (Kogawara et al. 2006). These accumulation patterns may be derived from species traits in sucrolysis and/or glycolysis under hypoxic conditions: it is presumed that an inhibition of sucrolysis may decrease substrate availability for glycolysis in hypoxic roots of E. camaldulensis but not of M. cajuputi.

We hypothesized that the ability to maintain sucrolytic activity associated with enhanced glycolytic activity determines the difference in tolerance to root hypoxia in these species. We first investigated the effect of hypoxia on root energy status and then examined the effects of feeding sugars, Glc or Suc, to the root on its energy status in hypoxia. We found that feeding of Glc but not Suc recovered the energy status of hypoxic roots of E. camaldulensis to the level of the control roots. Subsequently, we evaluated the response of sugar consumption rate (SCR), as an index of glycolysis/fermentation flux, to hypoxia by measuring the CO₂ production and O₂ consumption of roots. In order to investigate the metabolic background of the observed results, we examined the effects of hypoxia on the activities of sucrolytic and glycolytic enzymes and found that inhibition of sucrolysis may be a major factor in the energy status decline under hypoxia in E. camaldulensis.

Materials and methods

Plant materials and HT

Seeds of M. cajuputi were obtained from a lowland forest in Narathiwat Province (lat. 6°30′N, long. 101°45′E), Thailand. Seeds of E. camaldulensis (lot 19708) were kindly provided by the Australian Tree Seed Centre, CSIRO Forestry and Forest Products (Canberra, ACT, Australia). The seeds of each species were sown in acid-washed sand and germinated in a temperature-controlled greenhouse (30 °C for 16 h and 25 °C for 8 h; natural light). After germination, seedlings were grown for ~3 months with a nutrient solution supply as described in our previous study (Kogawara et al. 2006). Seedlings of a similar size of 10 cm in height were gently taken out from the seeding beds, and their roots were washed to remove the sand. The seedlings were transplanted into hydroponic culture in 12-L plastic containers filled with nutrient solution described previously (Kogawara et al. 2006). The containers were covered with PVC lids with holes to allow the shoots to protrude. The nutrient solution was aerated at 150 ml min⁻¹ and replenished every 3 days.

After acclimatization to the hydroponic culture for 1 month, seedlings were transplanted into new containers for HT. The sizes of the seedlings at the time of transplanting were 30 and 20 cm for M. cajuputi and E. camaldulensis, respectively. The O₂ concentration in the nutrient solution was controlled as previously described (Kogawara et al. 2006). Hypoxic treatment was initiated by bubbling N₂ through the solution at 150 ml min⁻¹. The nutrient solution for control seedlings was aerated as during the acclimatization period. The gas tightness of the containers was enhanced with sponges around the root collars of each seedling and with rubber tapes between the lids and the containers. The O₂ concentration was measured every day with a galvanic electrode (9520-10D, Horiba, Kyoto, Japan) connected to a dissolved O₂ meter (D-55, Horiba), and was within the same range as in our previous study, i.e., <0.25 mg l⁻¹ in HT and >8.0 mg l⁻¹ in the control.

Energy status of HT roots with or without sugar feeding

We analyzed the energy status of HT roots and then evaluated the effects of sugar feeding on root energy status to investigate...
whether substrate availability is the limiting factor for energy production in roots under hypoxia. Glucose or Suc was used for sugar feeding. Six containers, each holding five seedlings per species, were provided for the experiment. Among them, two containers (one per treatment) were used for non-feeding, and four containers (two per treatment) were used for sugar feeding. For each treatment with sugar feeding, one container was used for Glc feeding and the other one for Suc feeding. After 3 days of HT, Glc (2 mM) or Suc (1 mM) was dissolved in the nutrient solution for sugar feeding. To inhibit microbial propagation, the nutrient solution was circulated in a hose loop through a UV sterilizer (Turbo-Twist 3X, Energy Savers, Carson, CA, USA) for 15 min every hour.

At 4 days after HT, i.e., 1 day of sugar feeding, the lateral roots of each seedling were cut off, immediately frozen in liquid N\textsubscript{2}, freeze-dried and weighed. The samples (10–20 mg) were ground into powder with a mortar and pestle under liquid N\textsubscript{2}, followed by further grinding in 1.2 ml of 120 mM 5-sulfosalicylic acid. The homogenates were centrifuged at 17,500 g at 4 °C for 10 min. The supernatants were neutralized with 0.95 M KOH (107 µl) and centrifuged again. Debris and proteins in the supernatants were excluded by ultrafiltration on UFC3 LGC 00 filters (Millipore, Billerica, MA, USA). Filtrate aliquots (100 µl) were mixed with 100 mM KH\textsubscript{2}PO\textsubscript{4} (600 µl), and adenine nucleotides were derivatized to 1,N\textsuperscript{6}-ethenoadenine nucleotides by the addition of 45% chloroacetaldehyde (60 µl). The reactions were performed at 95 °C for 10 min. The derivatives were quantified by high-performance liquid chromatography as described by Yamanoshita et al. (2005). Each derivative (ATP, ADP, AMP) was separated by using an analytical reversed-phase column (5 µm ODS-C18, 150 mm × 6.0 mm internal diameter, Shimadzu, Kyoto, Japan), detected by a fluorescence detector (RF-10A, Shimadzu), using an excitation wavelength of 285 nm and an emission wavelength of 410 nm, and quantified on the basis of its peak area on the chromatogram. Adenylate energy charge was calculated as

\[
\text{AEC} = \frac{(\text{ATP} + 0.5\text{ADP})}{(\text{ATP} + \text{ADP} + \text{AMP})}
\]

**Root respiration**

Three seedlings of each species per treatment were prepared. At 4 days after HT was begun, the entire root system of each seedling (HT and control) was excised, and respiration was measured with an open portable photosynthesis system (LI-6400, LI-COR, Lincoln, NE, USA), which controlled the flow rate and CO\textsubscript{2} concentration in the inflow and measured the CO\textsubscript{2} concentration in the outflow. Figure 1a shows the outline of the measurement system. Respiration of HT roots was measured under hypoxic conditions, and that of control roots was measured under normoxic and hypoxic conditions. Each excised root (150–200 mg dry weight) was immediately immersed in 150 ml of the nutrient solution in a 180-ml wide-mouth glass jar, which was kept at 30 °C in a water bath.

![Figure 1. Outline of the measurement system for analyzing root CO\textsubscript{2} production rate (a) and O\textsubscript{2} consumption rate (b). IRGA, infrared gas analyzer; DO, dissolved oxygen.](https://academic.oup.com/treephys/article-abstract/34/3/229/244760)
The mouth of the jar was sealed with a rubber stopper, through which two gas tubes (for inflow and outflow) were inserted. The jar was set in the flow line between the pump and the infrared gas analyzer connected to the photosynthesis system. The tip of the inflow tube was immersed into the nutrient solution to aerate the solution, while the tip of the outflow tube was set above the solution surface. The inflow rate and CO$_2$ concentration were controlled by the photosynthesis system to be 300 µmol s$^{-1}$ and 350 µmol mol$^{-1}$, respectively. The air was supplied to the pump from a 50-ml tube to control the O$_2$ concentration in the inflow. For hypoxic measurements, N$_2$ gas was injected into the tube at 500 ml min$^{-1}$, resulting in 0.4–0.5 mg l$^{-1}$ O$_2$ in the inflow; no N$_2$ injection was used for normoxic measurements. The outflow from the jar passed through a cylinder filled with silica gel before entering the infrared gas analyzer. The CO$_2$ concentration in the inflow and outflow was measured over 20 min, and the CO$_2$ production rate was calculated from the difference and the flow rate. The difference in CO$_2$ concentration between the inflow and the outflow ranged from 5 to 10 µmol mol$^{-1}$.

Immediately after the CO$_2$ production rate was measured, the jar was detached from the flow line and filled with the nutrient solution to its maximum capacity and O$_2$ consumption rate was measured. Figure 1b shows the outline of the measurement system. The jar was sealed with a sponge, through which the dissolved O$_2$ meter was inserted into the solution. The decrease in O$_2$ concentration in the nutrient solution was monitored over 10 min at 30 °C. The O$_2$ consumption rate was calculated by linear regression at 0.4–0.5 mg l$^{-1}$ O$_2$. During the measurement, the solution was stirred with a magnetic stirrer to equilibrate the CO$_2$ and O$_2$ concentrations. After all measurements, each excised root was immediately microwaved and then oven-dried at 80 °C for 48 h to determine the dry weight. Carbon dioxide production and O$_2$ consumption rates are expressed as units per weight.

Assuming that CO$_2$ production from fermentation coincides with that from ethanol fermentation, we calculated the SCR of the respiratory metabolism from the O$_2$ consumption rate and the CO$_2$ release rate of each seedling as described previously (Albrecht et al. 2004). In the generation of CO$_2$ by Glc oxidation via glycolysis, we assumed that the tricarboxylic acid (TCA) cycle generates six molecules, and ethanol fermentation generates two molecules of CO$_2$ per Glc molecule. Simultaneously, six molecules of O$_2$ are consumed in the electron transport chain coupled to the TCA cycle. Thus, the fermentative CO$_2$ production rate and SCR in Glc basis were calculated as

$$\text{Fermentative CO}_2 \text{ production} = [\text{CO}_2]_{\text{total}} - [\text{O}_2]_{\text{total}}$$  \hspace{1cm} (1)

$$\text{SCR} = \frac{\text{Fermentative CO}_2 \text{ production}}{2} + \frac{[\text{O}_2]_{\text{total}}}{6}$$  \hspace{1cm} (2)

where [CO$_2$]$_{\text{total}}$ is the CO$_2$ release rate and [O$_2$]$_{\text{total}}$ is the O$_2$ consumption rate of total respiration.

**Glycolytic and fermentative enzyme assays**

Two containers (one for each treatment), each with five seedlings per species, were used. At 4 days after HT was applied, the lateral roots (HT and control) were cut off, immediately frozen in liquid N$_2$ and stored at −80 °C until enzyme extraction. Glycolytic and fermentative enzymes were extracted from the frozen samples with 1.5 ml of extraction buffer containing 50 mM HEPES–KOH (pH 7.5), 15 mM MgCl$_2$, 100 mM 2-mercaptoethanol, 2.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 15% (v/v) glycerol, 0.05% Triton X-100, 0.9% EDTA-free Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 6% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 17,500 g at 4 °C for 10 min, and the supernatants were desalted by centrifugation on Sephadex-G25 columns equilibrated with a buffer containing 50 mM HEPES–KOH (pH 7.5), 15 mM MgCl$_2$, 2.5 mM DTT, 1 mM PMSF and 15% (v/v) glycerol.

The activities of glucokinase (GK; EC 2.7.1.2), fructokinase (FK; EC 2.7.1.1), ATP:fructose-6-phosphate phosphotransferase (PFK; EC 2.7.1.11) and pyrophosphate (PPI):fructose-6-phosphate phosphotransferase (PFP; EC 2.7.1.90) were assayed in reaction mixtures (1 ml) containing 50 µl of the enzyme extracts according to Bouny and Saglio (1996) with modifications. For determination of GK or FK activity, the final reaction mixtures contained 50 mM HEPES–KOH (pH 7.5), 3 mM DTT, 2 mM MgCl$_2$, 1 mM ATP, 0.5 mM NADP$^+$, 1.2 units ml$^{-1}$ glucose-6-phosphate dehydrogenase, 4.8 units ml$^{-1}$ phosphoglucone isomerase and 1 mM Glc or fructose. The reactions were started by the addition of ATP. For determination of PFK activity, the final reaction mixtures contained 50 mM HEPES–KOH (pH 7.5), 5 mM fructose-6-phosphate, 2 mM MgCl$_2$, 1 mM ATP, 0.2 mM NADH, 1.2 units ml$^{-1}$ aldolase, 1.3 units ml$^{-1}$ glycerol-3-phosphate dehydrogenase and 8.4 units ml$^{-1}$ triose phosphate isomerase. The reactions were started by the addition of ATP. For determination of PFP activity, the final reaction mixtures contained 50 mM HEPES–KOH (pH 7.5), 5 mM fructose-6-phosphate, 2 mM MgCl$_2$, 1 mM PPI, 0.2 mM NADH, 5 µM fructose-2,6-biphosphate, 1.2 units ml$^{-1}$ aldolase, 1.3 units ml$^{-1}$ glycerol-3-phosphate dehydrogenase and 8.4 units ml$^{-1}$ triose phosphate isomerase. The reactions were started by the addition of PPI. Pyruvate kinase (PK; EC 2.7.1.40) activity was measured in the reaction mixtures (1 ml) containing 50 µl of the enzyme extracts and 60 mM Mops–NaOH (pH 7.2), 40 mM KCl, 10 mM MgCl$_2$, 1 mM ADP, 0.1 mM NADH and 4 units ml$^{-1}$ lactate dehydrogenase (Yamanoshita et al. 2005). The reactions were started by the addition of ADP. Alcohol dehydrogenase activity was measured in the reaction mixtures (1 ml) containing 50 µl of the enzyme extracts and 50 mM MES–NaOH (pH 6.0), 2 mM DTT, 6 mM MgCl$_2$, 0.1 mM NADH...
and 380 mM acetaldehyde (Kogawara et al. 2006). The reactions were started by the addition of acetaldehyde. All reactions were performed at 30 °C.

Enzyme activities were calculated from NADP reduction or NADH oxidation, monitored as the change in absorbance at 340 nm on a spectrophotometer (MPS-2450, Shimadzu). The protein concentration in each extract was determined after desalting by the Bradford (1976) method, and all enzyme activities were normalized to total protein.

**Sucrose cleavage assays**

Two containers (one for each treatment), each with four seedlings per species, were prepared. At 4 days after HT was applied, the lateral roots of each seedling (HT and control) were processed and stored as described for glycolytic and fermentative enzymes until enzyme extraction.

For the INV assay, the frozen samples were ground with an ice-cold mortar and pestle in 1.5 ml of the same extraction buffer as for glycolytic and fermentative enzymes. The homogenates were centrifuged at 17,500g at 4 °C for 10 min. The supernatants were used for the soluble acid INV assay. The pellets were washed three times with extraction buffer without PVPP, resuspended in the same buffer containing 500 mM NaCl and kept at 4 °C overnight to extract the cell-wall INV (Zhang et al. 2001). The homogenates were centrifuged at 17,500g at 4 °C for 10 min, and the supernatants were used for the cell-wall INV assay. Aliquots of each extract were desalted as above. Soluble acid INV and cell-wall INV were assayed in a total volume of 100 µl; each reaction mixture contained 100 mM sodium acetate buffer (pH 4.5 and 4.1, respectively), 20 mM Suc and 10 µl of the enzyme extract in 1.5-ml microtubes. The reaction was started by the addition of Suc, incubated for 30 min at 30 °C and then stopped by boiling for 1 min. Reducing sugars were determined by the Somogyi–Nelson method (Nelson 1944, Somogyi 1952). Mixtures with no incubation were used as blanks.

Sucrose synthase was extracted according to the method of Winter et al. (1997) with minor modifications. Frozen samples were ground with an ice-cold mortar and pestle in 1.5 ml of extraction buffer containing 50 mM Mops–NaOH (pH 7.5), 10 mM MgCl₂, 150 mM NaCl, 1 mM EDTA, 0.1 µM microcystin–LR, 100 mM 2-mercaptoethanol, 2.5 mM DTT, 1 mM PMSF, 15% (v/v) glycerol, 0.05% (v/v) Brij35 (Dojindo Laboratories, Kumamoto, Japan), 0.5% Complete protease inhibitor cocktail and 6% (w/v) PVPP. The homogenates were centrifuged at 10,000g at 4 °C for 20 min. The supernatants were transferred to new microtubes and ultracentrifuged at 100,000g at 4 °C for 1 h (Optima TLX, TLA-100.4 rotor, Beckman Coulter, Brea, CA, USA), and the supernatants were used as soluble SuSy fractions. The pellets were washed with the extraction buffer and solubilized in the same buffer plus 1% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The suspensions were ultracentrifuged at 100,000g at 4 °C for 1 h, and the supernatants were used as the SuSy microsomal fractions. Aliquots of each fraction were desalted by centrifugation on Sephadex-G25 columns equilibrated with a buffer containing 50 mM Mops–KOH (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 0.5 mM CaCl₂, 0.1 mM microcystin-LR and 15% (v/v) glycerol. Sucrose synthase activity was assayed as described by Zeng et al. (1998) with some modifications. The reaction was performed in a mixture (42 µl) containing 27 µl of the enzyme extract and 50 mM HEPES–KOH (pH 7.5), 15 mM MgCl₂, 10 mM fructose and 5 mM UDP–Glc at 30 °C for 30 min, and terminated by the addition of 42 µl of 30% KOH. To remove the unreacted fructose, we heated the mixtures at 100 °C for 10 min and then cooled them on ice. Sucrose synthase activity was calculated from the amount of Suc produced, which we quantified by adding 0.14% anthrone in H₂SO₄ and measuring absorbance at 620 nm. Mixtures with no incubation were used as blanks.

The protein concentration in each extract was determined as above, and all enzyme activities were normalized to total protein.

**Statistical analyses**

Statistically significant differences between treatments in the mean values of enzyme activities and root respiration rates were determined by the t-test. The SCR of control or HT roots under hypoxic conditions was compared with that of control roots under normoxic conditions by Dunnett’s test. The statistical differences in AEC and ATP concentrations among all combinations of treatments and sugar-feeding types were determined using Scheffé’s test. All statistical analyses were performed in the Excel Toukei software (version 6.0, Esumi, Tokyo, Japan).

**Results**

**Energy status of HT roots with or without sugar feeding**

After 4 days of HT without sugar feeding, AEC decreased <0.72 in _E. camaldulensis_ roots, but did not decrease in _M. caujuputi_ roots in comparison with normoxic roots (Figure 2a and b). The root ATP content was decreased by HT in _E. camaldulensis_ but not in _M. caujuputi_ (Figure 2c and d). These results indicate that _M. caujuputi_ can maintain its root energy status better than _E. camaldulensis_ upon several days of hypoxia.

To investigate whether low substrate availability for glycolysis is the cause of reduced energy in HT roots of _E. camaldulensis_, we compared the effects of Glc or Suc feeding on the energy status of HT and control roots of this species. Control roots fed with Glc or Suc showed AEC similar to unfed control roots (Figure 2a), suggesting that the sugar feeding did not have any effect on the root energy status. Hypoxic treatment roots fed with Glc showed similar AEC to control roots, but HT roots fed with Suc showed lower AEC than control roots (Figure 2a). The difference in the ATP content between unfed
control and HT roots was abolished by Glc feeding (Figure 2c). These results indicate that the shortage of substrate for glycolysis causes the lower energy status in HT roots of *E. camaldulensis*. In contrast, Suc feeding did not recover AEC to the level of the control roots. In the roots of *M. cajuputi*, the AEC and ATP content of HT roots was similarly high regardless of the sugar feeding (Figure 2b and d).

**Respiration rate and sugar consumption rate**

In both species, the O₂ consumption rate in control roots analyzed under hypoxic conditions was less than half that under normoxic conditions (Figure 3a), indicating that mitochondrial respiration was suppressed during the measurement. In both species, the O₂ consumption rate was similar in HT and control roots analyzed under hypoxic conditions (Figure 3a). Carbon dioxide production in control and HT roots was similarly suppressed during analysis under hypoxic conditions in *E. camaldulensis* (Figure 3b), whereas HT roots of *M. cajuputi* showed a slight (but not significant) increase in CO₂ production compared with control roots (Figure 3b). The difference between the CO₂ production rate and the O₂ consumption rate can be interpreted as the rate of CO₂ production from fermentation (Eq. (1); Figure 3c). In control roots of both species, fermentative CO₂ production increased when measured under hypoxia (Figure 3c). Fermentative CO₂ production in the HT roots was more than double that in control roots in *M. cajuputi* measured under hypoxia, but no such difference was observed in *E. camaldulensis* (Figure 3c).

Sugar consumption rate (Eq. (2)) in control roots under normoxia was compared with that in control or HT roots under hypoxia (Figure 4). In control roots of both species, SCR was lower under hypoxia than under normoxia. The responses of HT roots differed between the species. The SCR in *E. camaldulensis* HT roots was lower than that in control roots under normoxic conditions whereas that in *M. cajuputi* HT roots was similar to that in control roots under normoxic conditions.

**Activities of glycolytic enzymes**

In the roots of *E. camaldulensis*, the activities of all glycolytic enzymes investigated were not affected by HT (Table 1). In the roots of *M. cajuputi*, the activities of PFK and PK were increased upon HT, whereas those of the other glycolytic enzymes were not affected (Table 1). Alcohol dehydrogenase activity in HT roots was 4.6 times that in the control roots in *E. camaldulensis* and 3.1 times in *M. cajuputi* (Table 1), in line with our previous report (Kogawara et al. 2006).
Activities of sucrolytic enzymes

In both species, the activity of cytosolic SuSy was not altered by HT (Figure 5a), whereas the activity of membrane-associated SuSy was significantly increased in *E. camaldulensis*, but not in *M. cajuputi* (Figure 5b). The soluble acid INV activity was inhibited by HT in the roots of *E. camaldulensis*, but not in those of *M. cajuputi* (Figure 6a). The cell-wall INV activity was unaffected by HT in both species (Figure 6b).

Discussion

In our previous study using the same experimental system and species, we monitored photosynthesis and growth response during 19 days of HT (Kogawara et al. 2006). By the fifth day of HT, the photosynthesis of *E. camaldulensis* was reduced by stomatal closure. On the 10th day of HT and thereafter, the photosynthesis and growth of *E. camaldulensis* were inhibited almost completely, while no effect of HT was observed in *M. cajuputi* throughout the duration of the treatment (Kogawara et al. 2006). Both species showed induction of fermentation activity, evaluated by ADH activity, by the first day of HT. And at the third day of HT, both species showed different effects of HT on photoassimilate allocation (Kogawara et al. 2006). In the present study, we investigated the metabolic process that is responsible for the interspecies difference in tolerance to root hypoxia. We focused on the fourth day of HT, when metabolism is already affected but photosynthesis is still operating in *E. camaldulensis*.

The ability to maintain energy status and ATP content under root hypoxia was higher in *M. cajuputi* than in *E. camaldulensis* (Figure 2). Low energy status under O$_2$ deficiency can be...
responsible for a decrease in ion transport (Colmer et al. 2001) or root hydraulic conductivity (Tournaire-Roux et al. 2004), which is in line with the growth reduction of *E. camaldulensis* but not of *M. cajuputi* under root hypoxia (Kogawara et al. 2006). In the previous study investigating the effects of flooding on *M. cajuputi* roots, root AEC showed a drop to 0.7 on the second day of flooding and then recovered to the level of the control on the fourth day of the treatment (Yamanoshita et al. 2005), which is in agreement with the result in the present study. Both studies indicate that *M. cajuputi* is the species able to maintain normal energy status of roots when hypoxic stress is imposed.

The results showing that AEC recovered in HT roots of *E. camaldulensis* by feeding Glu revealed that substrate availability is the limiting factor of energy production in HT roots of this species. This is consistent with previous studies reporting that Glu feeding to hypoxic roots had a positive effect on survival or growth of root tips (Webb and Armstrong 1983, Bouny and Saglio 1996), suggesting that carbohydrates are the primary energy resource whose supply is more important for hypoxic roots than for well-aerated roots. The importance of carbohydrate reserves has also been proposed in studies on tree species, in which relatively flood-intolerant species reduced soluble sugar concentration (Kolb et al. 2002, Ferner et al. 2012) or starch concentration (Angelov et al. 1996, Gravatt and Kirby 1998) in flooded roots, but relatively flood-tolerant species kept them high. In contrast to Glu feeding, Suc feeding to HT roots of *E. camaldulensis* did not recover AEC (Figure 2a and c), implying that limited supply of Glc to glycolysis may be caused by impaired Suc degradation. Sucrose accumulation in HT roots in the species observed in the previous study (Kogawara et al. 2006) is in line with this possibility.

To investigate whether the activation of fermentation in HT roots differs between the two species, we measured root CO$_2$ production and O$_2$ consumption under hypoxia. Fermentative CO$_2$ production rate was similar in control and HT roots of *E. camaldulensis* whereas activation of fermentation was observed in HT roots of *M. cajuputi* (Figure 3c). Tolerance to O$_2$ deficiency can be improved with increasing ethanol production, which represents the increased fermentation (Bouny and Saglio 1996, Huang et al. 2003, Gharbi et al. 2007). The difference in the activation of fermentation in HT roots is the likely cause of the difference in tolerance to root hypoxia between *E. camaldulensis* and *M. cajuputi*.

These interspecies differences in fermentation can be interpreted in terms of sugar consumption. In roots grown under normoxic conditions, a sudden imposition of O$_2$ deficiency on them leads to a net decrease of respiratory sugar consumption because the aerobic respiration is restricted and fermentation is yet to be induced. This was shown in the SCR of the control
roots measured under hypoxic conditions, in both *M. cajuputi* and *E. camaldulensis* (Figure 4). Along with the de novo synthesis of fermentative enzymes, which is a part of the plant adaptive response to low O$_2$ (Chang et al. 2000), SCR is expected to increase with the growing demand for fermentation substrate. This is the case for HT roots of *M. cajuputi*, in which SCR was restored to the normoxic level (Figure 4) with the increased fermentation (Figure 3c). In contrast, *E. camaldulensis* failed to restore SCR in HT roots (Figure 4), which reflects the lack of an increase in fermentation (Figure 3c). Glycolytic flux in HT roots of *E. camaldulensis* seems not high enough to produce ATP through fermentation. Theoretically, low glycolytic flux in HT roots of *E. camaldulensis* could have resulted from an enzymatic impairment or substrate shortage. The recovery of energy status and ATP content in HT roots of this species by Glc feeding (Figure 2a and c) revealed that substrate shortage but not enzymatic impairment is the key to the low glycolytic flux in HT roots of this species. No inhibition of the activities of glycolytic enzymes in HT roots is in line with this conclusion (Table 1). Since we measured the enzymatic activities in vitro, some regulation in planta may affect the response manner of the enzyme activities to HT. However, this should not have limited the glycolytic flux in HT roots of *E. camaldulensis*, which was evidenced by the results of the Glc feeding.

The activities of several glycolytic enzymes increased in *M. cajuputi* roots (Table 1), similar to observations in other plant species (Rivoal et al. 1989, Fox et al. 1995, Bony and Saglio 1996). An increased turnover of carbohydrate incorporated into the soluble fraction in HT roots of this species, suggested by $^{13}$C experiments in the previous study (Kogawara et al. 2006), is consistent with this result. Nevertheless, the SCR value of HT roots under hypoxia was not superior to that of the control roots under normoxic conditions (Figure 3). These results imply that the glycolytic flux into pathways other than ethanol fermentation increased in *M. cajuputi* roots under hypoxia. In many plant species, the glycolytic flux into lactic acid fermentation is well known to increase in early response to O$_2$ deficiency (Davies et al. 1974, Roberts et al. 1984, Rivoal and Hans 1994). Lactic acid fermentation plays a role in regenerating NAD$^+$ to supply for glycolysis under O$_2$ deficiency, as well as ethanol fermentation (Greenway and Gibbs 2003). However, there are only a few cases in which lactate dehydrogenase activity persists for a long period after the induction of ethanol fermentation (Hoffman et al. 1986, Good and Crosby 1989). As for *M. cajuputi* roots, lactate dehydrogenase activity was not induced during the first week, while it was slightly induced during the second week (Yamanoshita et al. 2005). Thus, lactic acid fermentation is unlikely to be the reason for the higher tolerance of *M. cajuputi*. Alanine, a product of pyruvate metabolism, is also a major metabolite accumulated under O$_2$ deficiency (Ricoult et al. 2005, Kato-Noguchi 2006, Kreuzwieser et al. 2009). While it has been suggested that alanine production during anoxia may contribute to mitigate cytoplasmic acidification by competing with lactate production for pyruvate (Ricoult et al. 2005), evidence that alanine production directly affects energy status during O$_2$ deficiency has not been reported so far.

Since the glycolysis–fermentation pathway produces less ATP than aerobic respiration, a decrease in ATP production through inhibition of the TCA cycle cannot be compensated for by this pathway unless sugar consumption is increased (Gibbs and Greenway 2003). Despite no increase in SCR, HT roots of *M. cajuputi* sustained an energy status comparable to that of the control roots (Figure 2b). It is possible that the energy requirement for maintenance is reduced in HT roots of *M. cajuputi*, similar to anoxia-tolerant plant tissues such as rice coleoptiles (Colmer et al. 2001) and storage roots of red beet (Zhang and Greenway 1994). Generally in plant species, O$_2$ deficiency reduces protein synthesis, biosynthetic processes that have a high energy cost (Edwards et al. 2012), but often simultaneously increases the net synthesis of specific proteins (Sachs et al. 1980, Mocquot et al. 1981, Ricard and Pradet 1989), so-called anaerobic proteins (ANPs). Many of the ANPs are enzymes of glycolysis and fermentation, presumably related to induced activities of these enzymes as observed in *M. cajuputi* (Table 1). In addition to these classical ANPs, some other proteins whose synthesis is enhanced under anoxia were detected in organs that can grow under anoxia (Ishizawa et al. 1999, Huang et al. 2005), including enzymes involved in the PPI-dependent metabolism and scavenging system of reactive oxygen species (Huang et al. 2005). Such specific protein synthesis allows plant cells to use energy efficiently for maintenance, resulting in a reduction of energy requirement (Greenway and Gibbs 2003). This could also be the case for HT roots of *M. cajuputi*.

Previous reports have indicated that the sustained activity of carbohydrate-degrading enzymes to ensure hexose entry into respiratory metabolism is a key for energy production under O$_2$ deficiency. A difference in tolerance of two oak species to waterlogging could be attributed to their different abilities to maintain glycolysis and fermentation, reflected in the upregulation of mRNA levels of not only glycolytic and fermentative enzymes but also amylase, which degrades starch to soluble carbohydrates (Le Provost et al. 2012). Cereal seeds with dysfunctional amylases cannot germinate under anoxia, and benefit in this respect from exogenous Glc (Perata et al. 1992), which suggests that the inability to use carbohydrate reserves for glycolysis under anoxia is critical for energy production for seed germination. Amylase activity has been suggested to play a key role in the maintenance of glycolytic flux into ethanolic fermentation in elongating rice coleoptiles under anoxia (Huang et al. 2003), and variation in amylase activity was correlated with variation in anoxia tolerance among rice cultivars (Ismail et al. 2009). As well as the role of starch
degradation during O₂ deficiency, Suc degradation must be sustained to supply substrate for anaerobic energy metabolism, especially when carbohydrate supply depends mainly on photoassimilate transport from the shoot. This situation was demonstrated by our present study using intact roots with sugar feeding (Figure 2), and was the reason for analyzing activities of Suc-degrading enzymes, SuSy and INV.

To investigate the background of the impaired degradation of Suc in HT roots of *E. camaldulensis*, we measured the activities of soluble and insoluble INV and SuSy. Since Suc degradation via SuSy saves ATP in comparison to its degradation via INV when its products are subsequently metabolized in glycolysis (Stitt 1998, Koch 2004), the induction of SuSy (in particular, its cytosolic form) has been recognized as an adaptive response to low-O₂ conditions (Guglielminetti et al. 1997, Zeng et al. 1999). However, HT did not affect the activity of cytosolic SuSy in either species (Figure 5a), while increasing the activity of membrane-associated SuSy in *E. camaldulensis* (Figure 5b). Membrane-associated SuSy plays a role mainly in directing carbon to polysaccharide synthesis (Amor et al. 1995). The activity of membrane-associated SuSy was increased and was accompanied by callose deposition in anoxic maize root tips (Subbaiah and Sachs 2001), or by cellulose deposition in hypoxic wheat roots (Albrecht and Mustroph 2003). Thus, the induction of membrane-associated SuSy under hypoxia could reduce substrate availability for glycolysis. However, at least in *E. camaldulensis*, this effect would be negligible, since the previous study showed that the incorporation of ¹³C-labeled photoassimilate into the structural carbohydrate, the corresponding fraction for cellulose and callose, was not increased during HT (Kogawara et al. 2006). Thus, SuSy seems not involved in the impaired degradation of Suc in HT roots of *E. camaldulensis*.

Soluble acid INV activity in *E. camaldulensis* was reduced by HT (Figure 6a), which is in agreement with observations for other plant species (Zeng et al. 1999, Albrecht and Mustroph 2003). Soluble acid INV is located in the vacuole in most plant species and plays a pivotal role in hexose entry into cytoplasmic metabolism (Winter and Huber 2000, Koch 2004, Bocock et al. 2008). Inhibition of soluble acid INV in HT roots of *E. camaldulensis* (Figure 6a) may be responsible for the limited substrate availability for respiratory metabolism in HT roots. The accumulation of root Suc observed in our previous study (Kogawara et al. 2006) may result from inhibition of this enzyme. Sustained activity of soluble acid INV under HT in *M. cajuputi* (Figure 6) would provide an advantage to this species by keeping the substrate availability for respiratory metabolism at a sufficient level. While the behavior of SuSy has been extensively studied as a factor involved in the mechanism of low O₂ tolerance (e.g., Guglielminetti et al. 1997, Ricard et al. 1998, Zeng et al. 1998, 1999, Kumutha et al. 2008), INV has not been previously reported as a determinant of low O₂ tolerance. Because SuSy has a role in saving ATP as mentioned above, and INV participates in futile cycling of sugars that consume considerable ATP (Dieuaide-Noubhani et al. 1995, Nägele et al. 2010), INV repression accompanied by cytosolic SuSy induction has an advantage of avoiding energy waste under low O₂ conditions. However, our results suggest that, in the case when cytosolic SuSy is not induced, stability of INV is crucial for maintenance of energy metabolism under low O₂ conditions. The adaptive response of SuSy for anaerobic energy metabolism is not universal among plant species, as another role of SuSy in carbon partitioning under low O₂ condition has been found (Subbaiah and Sachs 2001, Albrecht and Mustroph 2003). Therefore, a comprehensive analysis of SuSy and INV is important for understanding the underlying causes of interspecies difference in hypoxia tolerance.

In conclusion, our data are consistent with the hypothesis that the difference in stable sucrolytic activity (mainly due to soluble acid INV) and hence in substrate availability for glycolysis determines the difference in energy metabolism and in tolerance to root hypoxia between *M. cajuputi* and *E. camaldulensis*. In *E. camaldulensis*, the decline in substrate availability is likely to be the main cause of the failure to activate fermentation in response to hypoxia. Sustained sucrolytic enzyme activities in HT roots of *M. cajuputi* should be a prerequisite for maintaining high energy status under hypoxia.

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Conflict of interest
None declared.

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