Sugar-Mediated Acclimation: The Importance of Sucrose Metabolism in Meristems

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We have designed an in vitro experimental setup to study the role of sucrose in sugar-mediated acclimation of banana meristems using established highly proliferating meristem cultures. It is a first step toward the systems biology of a meristem and the understanding of how it can survive severe abiotic stress. Using the 2D-DIGE proteomic approach and a meristem-specific EST library, we describe the long-term acclimation response of banana meristems (after 2, 4, 8, and 14 days) and analyze the role of sucrose in this acclimation by setting up a control, a sorbitol, and a sucrose acclimation treatment over time. Sucrose synthase is the dominant enzyme for sucrose breakdown in meristem tissue, which is most likely related to its lower energy consumption. Metabolizing sucrose is of paramount importance to survive, but the uptake of sugar and its metabolism also drive respiration, which may result in limited oxygen levels. According to our data, a successful acclimation is correlated to an initial efficient uptake of sucrose and subsequently a reduced breakdown of sucrose and an induction of fermentation likely by a lack of oxygen.

Keywords: Plant proteomics • abiotic stress • meristems • 2DDIGE • multivariate statistics • sucrose metabolism • EST library

Introduction

Acclimation is the process of an organism adjusting to a change in its environment, allowing it to survive future severe changes in temperature, water, and/or nutrient availability, and is a quite complex process. In plant cells, sucrose plays an important role in acclimation. In general, abundant carbon resources repress the expression of genes involved in photosynthesis and reserve mobilization but favor genes involved in carbon metabolism and storage and trigger other classes such as defense genes, secondary product pathways, and storage proteins.1 The cytosolic glycolytic network furnishes plants with the metabolic options needed for development and acclimatization to environmental stresses.2 In sink tissues, sucrose can be metabolized two ways: via invertase and via sucrose synthase (SUS). In Arabidopsis a significant proportion of the sucrose is metabolized via invertase rather than SUS, whereas in crop species it is likely that selection during domestication for high-yielding sink organs has led to a situation in which SUS is the dominant enzyme.3 A full understanding of this complex gene family and its role in acclimation is not yet acquired, and it is clear that a big discrepancy exists between the model Arabidopsis and crops.

The design of a laboratory setup to study acclimation in a crop and screen the current biodiversity under controlled conditions is extremely important. We (K.U.Leuven) host the Musa Bioversity International germplasm collection (>1200 accessions) as an in vitro and cryopreserved collection.5 With an annual production of about 120 million tons, banana and plantain are one of the most important food commodities after rice, wheat, and maize. Bananas and plantains are cultivated in more than 120 countries and are a staple food source of 400 million people. We have developed an in vitro setup to screen the Musa biodiversity for sugar-mediated acclimation in the framework of the cryopreservation of the collection. Meristems are subjected to an osmotic acclimation treatment prior to severe dehydration and subsequent freezing in liquid nitrogen. After thawing, their ability to regenerate in relation to the osmotic acclimation treatment is determined. This setup is analogous to the more generally utilized seed germination assay. Since most of the edible banana varieties are sterile, it provides a quick assay of response and is a very efficient system to screen the biodiversity of our collection.

We have previously shown that an acclimation mediated by sucrose is essential for the meristems to survive the severe...
dehydration treatment associated with cryopreservation. Su-
Strosse and co-workers. The meristem cultures were kept on
istem cultures were initiated and maintained as described by
regeneration, 5 batches containing 6
sorbitol. All cultures were kept in the dark at 25 °C.

Cryopreservation and evaluation of the regeneration was
eexecuted as described by Panis and co-workers. Briefly,
climated meristems are subjected to a severe dehydration
prior to freezing in liquid nitrogen. A high water content prior
t o freezing is lethal due to the formation of ice crystals. After

Using the 2D-DIGE proteomic approach, we characterized
the long-term recovery acclimation response of the meristems.
To understand the importance of including sucrose as a sugar
in the acclimation medium and to dissect the different aspects of
sucrose-mediated acclimation, we have set up a control, a
sorbitol, and a sucrose treatment over time. We unraveled for
the first time the different aspects of sugar-mediated acclimation
of meristems by monitoring hundreds of proteins over time in
a kinetic proteome study. This report is a first step toward an
insight into the systems biology of a meristem under osmotic
stress and combines proteomics and transcriptomics data to
characterize the meristematic tissue.

Materials and Methods

Plant Material. In vitro plants were provided by the Biover-
sity International Musa collection at K.U.Leuven, Belgium. The
selected variety Cachaco (ITC 0643 cooking banana) belongs to
the ABB genomic group, which is known to be tolerant
toward drought and cryopreservation. Multiple shoot mer-
istem cultures were initiated and maintained as described by
Strosse and co-workers. The meristem cultures were kept on
different media for 14 days: (i) the standard control multiple shoot meristem medium (this medium contains 0.09
M sucrose), (ii) the standard medium with increased level of
sucrose (0.4 M = 0.09 + 0.31 M), and (iii) the standard medium
complemented with 0.31 M sorbitol (0.09 M sucrose + 0.31 M
sorbitol). All cultures were kept in the dark at 25 ± 2 °C.

Acclimation Experiment. During the different acclimation
treatments a differential response in shoot regeneration after
dehydration and freezing was observed that could be correlated
with sucrose is more powerful than the sorbitol treatment
was moreover a strong indication that the osmotic treatment
in the acclimation medium and to dissect the different aspects
of sucrose-mediated acclimation, we have set up a control, a
sorbitol, and a sucrose treatment over time. We unraveled for
the first time the different aspects of sugar-mediated acclimation
of meristems by monitoring hundreds of proteins over time in
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to freezing is lethal due to the formation of ice crystals. After
thawing, their ability to regenerate in relation to the osmotic
acclimation treatment is determined. For the evaluation of
the regeneration, 5 batches containing 6–11 meristem clumps
were evaluated for each treatment and each time point.

Protein samples were taken after 2, 4, 8, and 14 days
of treatment. Acclimation was considered successful when mer-
istem survivors the severe dehydration and subsequent freezing
at −196 °C and were able to regenerate into shoots.

Protein Extraction and 2DE. Samples were extracted and
separated according to the phenol extraction method described
by Carpentier and co-workers. Proteins were labeled with Cy2,
Cy3, and Cy5 (GE-Healthcare, Diegem, Belgium) and scanned
as described by Carpentier and co-workers.

Statistics. For protein analysis, Decyder 7.0 software (GE-
Healthcare) and its EDA module were used for the ANOVA
(false discovery rate and multiple comparison analysis) and
principal component analysis (PCA). PCA was performed on
the spots occurring in at least 75% of all the internal standard
spot maps. The individual PCA loading and score plot data were
exported from Decyder as an xml file. For a more detailed
description of the statistical proteomics workflow, see Carpen-
tier and co-workers.

ANOVA and Tukey HSD analysis of the regeneration data
was performed with Statistica software (Statsoft).

Protein Identification and EST Annotation. Protein identi-
fication is based on peptide homology analysis of mass spectrometry derived peptide sequences. The sequence from
protein isolation to protein identification is essentially per-
sformed by the NCBI BLAST interface. Identifications obtained by the NCBI Virdiplantae sequence data set and the MUSA EST data set were blasted in batch
against the Swissprot database and NCBI Nr using the blastcl3
tool, which interacts directly with the NCBI BLAST ser-
ver (http://www.ncbi.nlm.nih.gov/BLAST/blast.html). All details of the identifications are listed in Table S5 in
Supporting Information. The ProtParam tool of the ExPasy
server (http://ca.expasy.org/) was used to calculate the grand
average of hydropathicity (GRAVY) score and other parameters
such as the theoretical pI and M.W. The number of transmem-
brane domains was calculated by the TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/). Sequence alignments
were performed using the ClustalW2 tool of the European
Bioinformatics Institute (http://www.ebi.ac.uk/Information/).

Generation of EST Libraries. Total RNA was purified from
the samples using the RNeasy Plant Mini Kit (QiAGEN, www.
qiagen.com, protocol: Purification of total RNA from plant cells
and tissues and filamentous fungi). mRNA were then isolated
using the Micro-Fast Track 2.0 Kit (Invitrogen, www.invitro-
gen.com, protocol: mRNA isolation from total RNA + Basic
mRNA isolation method). The mRNA was then used to produce
cDNA libraries using the Creator SMART cDNA Library Con-
struction Kit (Clontech Laboratories, www.clontech.com, protocol:
SMART cDNA synthesis by LD PCR + Ligation of ds
cDNA to pDNR-LIB). The Prime Script Reverse Transcriptase
(Takara Bio Inc., www.takara-bio.co.jp) and the Advantage 2
Ligation of ds

Results

Acclimation Experiment. During the different acclimation
treatments a differential response in shoot regeneration after
dehydration and freezing was observed that could be correlated
to protein changes. A two-way analysis of variance (α ≤ 0.01)
showed significant differences among the acclimation treat-
ments and among the time points and a clear interaction
between the factors time and treatment (Figure 1). At 4 days,
the two osmotic treatments were significantly different from
the control treatment (Tukey HSD test, α ≤ 0.05), and there
was moreover a strong indication that the osmotic treatment
with sucrose is more powerful than the sorbitol treatment
(Figure 1).
Quantitative Proteomics. Using the 2D-DIGE proteomic approach, we characterized the proteome of the control treatment, the sorbitol treatment, and the sucrose treatment on day 4 to correlate the treatment differences and acclimation to specific proteins. Principal Component Analysis (PCA) showed that the proteomes of the control, sorbitol, and sucrose acclimation treatment were different on day 4 (Figure 2A). The first two principal components explain 62% of the variance.

On the basis of their PCA loadings, 78 protein spots were positively correlated to the sucrose treatment (sucrose high, Figure 2B, first quadrant), 86 protein spots were positively correlated to the sorbitol treatment (sorbitol high (or osmotic stress high), Figure 2B, second quadrant), 77 protein spots were negatively correlated to the sucrose treatment (sucrose low, Figure 2B, third quadrant), and 94 protein spots were positively correlated to control treatment (osmotic stress low, Figure 2B, fourth quadrant). Respectively, 47, 52, 47, and 64 protein spots were significant after ANOVA FDR analysis ($R < 0.05$). PCA and ANOVA are two important ways to characterize the proteome.\textsuperscript{15}

The protein spots that were selected via both statistical methods and that could confidently be identified via MS/MS are listed in Table S1 in Supporting Information. Since our interest was to understand the role of sucrose metabolism, we focused on the proteins related to glycolysis (Table 1). To understand why shoot regeneration was highest at day 4, we subsequently characterized the proteome of the sucrose

Figure 1. Acclimation experiment, an in vitro setup. Banana meristems were subjected to a control, sorbitol, and sucrose acclimation treatment for 2, 4, 8, and 14 days, and the percent of shoot regeneration was determined after dehydration and freezing. Differences were evaluated by two-way analysis of variance ($n = 5$) and post hoc evaluation Tukey HSD ($\alpha < 0.05$). Bars marked with the same letter do not differ significantly from each other; % shoot regeneration $a < b < c < d < e$.

Figure 2. 2D DIGE experiment multivariate analysis. (A) Score plot. (B) Loading plot (proteins significantly different by ANOVA FDR are indicated in blue). Via the score plot and loading plot, proteins can specifically be correlated to the sucrose acclimation treatment (first (upper right) quadrant and third (lower left) quadrant), to the sorbitol acclimation treatment (second (lower right) quadrant), and to the control treatment (fourth (upper left) quadrant). The first two principal components explain 62% of the variance.
treatment in time to correlate the optimal treatment time (day 4) to specific proteins. PCA confirmed that indeed the proteome changed over time (Figure S1 in Supporting Information). The first two principal components explain 53.3% of the variance. PC1, explaining 40% of the variance, could be correlated to the length of the treatment. The analysis of the sucrose treatment at the time points 2, 4, 8, and 14 days clearly showed a time effect with a difference between the early phase (positively correlated to 2 and 4 days, 367 protein spots) and the late phase of acclimation (positively correlated to 8 and 14 days, 442 protein spots). In total, 42 protein spots were significant after ANOVA FDR analysis ($\alpha < 0.05$). Though some individual protein spots could be correlated to 4 days, a general correlation to the optimal acclimation treatment time (4 days) could not be shown. The protein spots that were selected via both statistical methods and that could confidently be identified via MS/MS are listed in Table S2 in Supporting Information. The proteins related to glycolysis are listed in Table 2.

To understand the osmotic stress aspect of the acclimation response in time, we also characterized the proteome of the sorbitol treatment on days 2, 4, 8, and 14 to correlate the optimal treatment time (day 4) to specific protein spots. PCA visibly indicated that the proteome changed over time (Figure 3). The first two principal components explain 59.0% of the variance.

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### Table 1. Identified Significant Proteins Taken at 4 Days of Treatment Belonging to the Glycolysis Pathway

<table>
<thead>
<tr>
<th>ID no.</th>
<th>annotation via the closest homologue</th>
<th>PCA classification</th>
<th>ANOVA (FDR)</th>
<th>multiple comparison treatment</th>
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<tr>
<td>5814</td>
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<td>5608</td>
<td>alcohol dehydrogenase</td>
<td>osmotic stress high</td>
<td>0.0000</td>
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<td>5359</td>
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<td>osmotic stress high</td>
<td>0.0001</td>
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<td>484</td>
<td>phosphoenolpyruvate carboxylase</td>
<td>osmotic stress high</td>
<td>0.0059</td>
<td>a a b</td>
</tr>
<tr>
<td>5158</td>
<td>SUS</td>
<td>osmotic stress high</td>
<td>0.0007</td>
<td>b a c</td>
</tr>
<tr>
<td>3319</td>
<td>enolase</td>
<td>sucrose high</td>
<td>0.0000</td>
<td>a c b</td>
</tr>
<tr>
<td>3126</td>
<td>enolase</td>
<td>sucrose high</td>
<td>0.0008</td>
<td>a b b</td>
</tr>
<tr>
<td>4439</td>
<td>enolase</td>
<td>sucrose high</td>
<td>0.0094</td>
<td>a b b</td>
</tr>
<tr>
<td>4956</td>
<td>phosphoenolpyruvate carboxylase</td>
<td>sucrose high</td>
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<td>a b b</td>
</tr>
<tr>
<td>2903</td>
<td>phosphoglycerate kinase</td>
<td>sucrose high</td>
<td>0.0001</td>
<td>a b c</td>
</tr>
<tr>
<td>4556</td>
<td>phosphoglyceromutase</td>
<td>sucrose high</td>
<td>0.0004</td>
<td>a c b</td>
</tr>
<tr>
<td>5615</td>
<td>pyruvate decarboxylase</td>
<td>sucrose high</td>
<td>0.0043</td>
<td>a ab b</td>
</tr>
<tr>
<td>4922</td>
<td>SUS</td>
<td>sucrose high</td>
<td>0.0001</td>
<td>a b a</td>
</tr>
<tr>
<td>5043</td>
<td>SUS</td>
<td>sucrose high</td>
<td>0.0009</td>
<td>a b b</td>
</tr>
<tr>
<td>5090</td>
<td>SUS</td>
<td>sucrose low</td>
<td>0.0000</td>
<td>b a a</td>
</tr>
<tr>
<td>4703</td>
<td>SUS</td>
<td>sucrose low</td>
<td>0.0001</td>
<td>c b a</td>
</tr>
<tr>
<td>4834</td>
<td>SUS</td>
<td>sucrose low</td>
<td>0.0001</td>
<td>c b a</td>
</tr>
<tr>
<td>5615</td>
<td>pyruvate decarboxylase</td>
<td>sucrose low</td>
<td>0.0003</td>
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<tr>
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<td>sucrose low</td>
<td>0.0055</td>
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<tr>
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<td>sucrose low</td>
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<tr>
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<td>SUS</td>
<td>osmotic stress low</td>
<td>0.0001</td>
<td>b c a</td>
</tr>
</tbody>
</table>

* $\text{su}c = \text{sucrose treatment, sorb = sorbitol treatment, cont = control treatment. Significance: } \alpha < 0.05$. Abundance: a > b > c; results with the same letter indicate no significant difference in abundance.

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### Table 2. Identified Significant Proteins Taken at 2, 4, 8, and 14 Days of Sucrose Treatment Belonging to the Glycolysis Pathway

<table>
<thead>
<tr>
<th>ID no.</th>
<th>annotation via the closest homologue</th>
<th>PCA classification</th>
<th>ANOVA (FDR)</th>
<th>multiple comparison (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5615</td>
<td>pyruvate decarboxylase</td>
<td>early phase</td>
<td>0.0048</td>
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</tr>
<tr>
<td>3372</td>
<td>pyruvate orthophosphate dikinase</td>
<td>early phase</td>
<td>0.0013</td>
<td>a a b c</td>
</tr>
<tr>
<td>4703</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0029</td>
<td>a b c c</td>
</tr>
<tr>
<td>5461</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0112</td>
<td>a b b b</td>
</tr>
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<td>5232</td>
<td>SUS</td>
<td>early phase</td>
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<td>a b b b</td>
</tr>
<tr>
<td>5090</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0283</td>
<td>a b b b</td>
</tr>
<tr>
<td>5043</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0330</td>
<td>ab a a b</td>
</tr>
<tr>
<td>4834</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0493</td>
<td>a ab b b</td>
</tr>
<tr>
<td>4578</td>
<td>SUS</td>
<td>early phase</td>
<td>0.0497</td>
<td>a ab b b</td>
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<td>alcohol dehydrogenase</td>
<td>late phase</td>
<td>0.0046</td>
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<tr>
<td>4415</td>
<td>phosphoglucomutase</td>
<td>late phase</td>
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<td>b c bc b</td>
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<td>4969</td>
<td>phosphoglycerate kinase</td>
<td>late phase</td>
<td>0.0083</td>
<td>c b b a</td>
</tr>
<tr>
<td>4540</td>
<td>phosphoglycerate kinase</td>
<td>late phase</td>
<td>0.0133</td>
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</tr>
<tr>
<td>2903</td>
<td>phosphoglycerate kinase</td>
<td>late phase</td>
<td>0.0138</td>
<td>b b a a</td>
</tr>
<tr>
<td>3935</td>
<td>phosphoglycerate kinase</td>
<td>late phase</td>
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<td>c bc ab a</td>
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<tr>
<td>4556</td>
<td>phosphoglyceromutase</td>
<td>late phase</td>
<td>0.0063</td>
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<td>phosphoglyceromutase</td>
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<td>0.0067</td>
<td>b b a a</td>
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<td>SUS</td>
<td>late phase</td>
<td>0.0365</td>
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<td>triose phosphate isomerase</td>
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<td>3865</td>
<td>UGPase</td>
<td>late phase</td>
<td>0.0044</td>
<td>b b b a</td>
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</table>

* Significance $\alpha < 0.05$. Abundance: a > b > c; results with the same letter indicate no significant difference in abundance.
PC1, explaining 43.7% of the variance, can be correlated to time. A kinetic proteome analysis clearly showed a time effect with a big difference between early phase 250 protein spots (positively correlated to 2 and 4 days) and late phase 368 protein spots (positively correlated to 8 and 14 days). Contrary to the sucrose treatment, a general correlation to the optimal acclimation time of 4 days could be revealed (Figure S2 in Supporting Information). One hundred ninety-four of the 250 early phase protein spots were specifically correlated to a high abundance at 4 days. In total, 131 protein spots were significant after ANOVA FDR analysis ($R_{e}0.05$). The protein spots that were selected via both statistical methods and that could confidently be identified via MS/MS are listed in Table S3 in Supporting Information. The proteins related to glycolysis and the pentose phosphate pathway are listed in Table 3.

Characterization of the Meristems. To characterize the meristems further, we constructed a tissue-specific EST library. After filtering and cleanup, the library contained 11070 reads, which have been assembled into 1433 contigs. Preparative 2DE gels were run from the same batch of samples, and the 1000 most abundant proteins were picked for identification (Figure S3A and B in Supporting Information; odd spots can be found in Figure S3A, even spots in Figure S3B). In total, 648 protein spots could successfully be identified, of which 322 were identified via the EST libraries (Table S4 in Supporting Information). Seventy-five protein spots were identified exclusively via the EST libraries, which is 12% of the identified spots. Two hundred forty-seven protein spots were identified both via cross species identification and the EST libraries. To determine the complementarity of the EST and 2DE characterization, we predicted via the closest homologue the number of transmembrane domains, $p_f$ and $M_i$ for both methods. The EST libraries cover genes coding for proteins with a broad $p_f$ and $M_i$ range (respectively, 4–13 and 1000–234000), while the 2DE approach is restricted to the chosen $p_f$ (4–7) and proteins that are too big or too small are not quantified (range 11000–171000).

#### Table 3. Identified Significant Proteins Taken at 2, 4, 8, and 14 Days of Sorbitol Treatment Belonging to the Pentose Phosphate (Bold) and the Glycolysis Pathway

<table>
<thead>
<tr>
<th>ID no.</th>
<th>annotation via the closest homologue</th>
<th>PCA classification</th>
<th>ANOVA (FDR)</th>
<th>multiple comparison (days)**</th>
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<td>5354</td>
<td>6-phosphogluconate dehydrogenase</td>
<td>4 days high</td>
<td>0.0007</td>
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<tr>
<td>5635</td>
<td>6-phosphogluconate dehydrogenase</td>
<td>4 days high</td>
<td>0.0035</td>
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<td>4533</td>
<td>aldo/keto reductase</td>
<td>4 days high</td>
<td>0.0112</td>
<td>b a b b</td>
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<tr>
<td>5606</td>
<td>glu-6-phosphate 1-dehydrogenase</td>
<td>4 days high</td>
<td>0.0220</td>
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<td>transketolase-like protein</td>
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<td>5615</td>
<td>pyruvate decarboxylase</td>
<td>early phase</td>
<td>0.0003</td>
<td>a a b c</td>
</tr>
<tr>
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<td>pyruvate dikinase</td>
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<td>a b b b</td>
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<tr>
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<td>phosphoglycerate mutase</td>
<td>late phase</td>
<td>0.0346</td>
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</tr>
<tr>
<td>4578</td>
<td>SUS</td>
<td>late phase</td>
<td>0.0414</td>
<td>bc c ab a</td>
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</table>

**Significance $R_{e0.05}$. Abundance: a > b > c > d; results with the same letter indicate no significant difference in abundance.
A wide range of varieties all differing in response and in severe dehydration in meristems. In previous reports, we evaluated osmotic acclimation and to study tolerance toward a meristem cultures proved to be an attractive approach to mRNA and protein levels and that post-translational modifications (PTM) complicate the interpretation. Seventeen different spots were identified as SUS (Table S4 in Supporting Information) decreases over time and that 1 of the 2 isoforms is highly abundant at 4 days and 8 days of treatment (Table 2: 5043). The highest measured abundance for spot 5043 was at 4 days (data not shown).

Although the low abundance of the different SUS isoforms was specifically correlated to the sucrose acclimation treatment, in the sorbitol treatment also 3 of the 7 isoforms were significantly less abundant compared to the control treatment (Table 1: 4703, 4834, 5480), indicating that osmotic stress in general could also play a role. Osmotic stress could induce initially a sucrose metabolism, generating a lot of metabolites, which might be responsible for the feedback of SUS. The analysis of the sorbitol treatment over time shows that SUS has a more or less constant level over time, but there is a trend showing that SUS has the lowest abundance at 4 days of acclimation (Figure S7 in Supporting Information). Though only one spot is significantly different (Table 3, spot 4578), 6 other SUS spots (4703, 4834, 4989, 5090, 5232, 5461) show the same trend and are all correlated in the PCA analysis.

These findings confirm that the lower abundance of SUS is mainly sucrose-specific and confirm our previous hypothesis that a lower level of SUS is correlated with a better acclimation by creating an osmoprotective advantage of an endogenous high-sucrose environment since the optimal acclimation time (4 days of acclimation) is correlated with a lower SUS abundance (Table 2, Table 3). This is an indication that sucrose plays an important role during acclimation in meristems and might moreover explain a difference in acclimation efficiency between the sorbitol and sucrose treatment. The initial sucrose concentration was much higher in the sucrose-treated meristems compared to the sorbitol-treated meristems (respectively 0.4 M and 0.09M).

Balance between Respiration and Fermentation. Oxygen supply into plant tissues can sometimes be problematic. Oxygen can fall rapidly to low levels within metabolically active, dense, or bulky plant tissues, even when external oxygen concentrations are high. The oxygen access in our metabolically highly active meristem tissue might indeed be an issue. This could explain why SUS is the only enzyme identified in both the proteome and transcriptome analysis. As stated above, SUS is the predominant enzyme for sucrose breakdown in sink tissues of crops, and this is related to its lower energy consumption. The two pathways of sucrose degradation to hexosephosphates differ in their energy costs. The breakdown of one molecule of sucrose via invertase requires two molecules of ATP, whereas the breakdown via SUS and UGPase requires only one molecule of PPi. The overall energy cost of the SUS pathway is even lower if one assumes that PPi is produced as a byproduct in many biosynthetic reactions. The effect of low internal oxygen concentrations has been investigated by Bologa and co-workers. This group concluded that declining internal oxygen levels can lead to a decrease in adenylate levels and a subsequent switch to pathways that consume less ATP. They pointed out that saving ATP could be an important metabolic adaptation to decreased oxygen consumption and prevent the tissue from driving itself into anoxia.

In our study we cover nearly all the enzymes of the initial and energy conserving phase of glycolysis. Since we did not identify the ATP-dependent phosphofructokinase in either the EST or proteomics data and since we did identify the PPi-
dependent isoform, we believe that there is a bias toward pathways that consume less ATP in our tissue. Additionally, we see that the abundance of SUS decreases especially in the sucrose acclimation treatment and that proteins belonging to the fermentation pathway are more abundant in the stress treatments (Table 1). Our hypothesis is that the internal oxygen concentration is even under control conditions an issue, which would explain why SUS is the only sucrose metabolizing enzyme in our meristem setup. In general, stresses cause high energy consumption and enhance the respiration.28,29 Furthermore, the uptake of sugars and their metabolism also consumes ATP.30 A high sucrose concentration leads initially to a high sucrose uptake, a high sucrose breakdown, and a higher amount of breakdown products. The uptake of sugar and its metabolism consumes ATP and drives respiration,30 resulting in limited oxygen levels, which might finally lead to anoxia. A lower level of SUS is probably a (feedback) mechanism controlling the level of sucrose and its breakdown products. Recently, Zabalza and co-workers observed that feeding pyruvate to pea roots attenuated signals that regulate fermentative activity in response to low oxygen and led to an increase in oxygen consumption and finally to anoxia.31 They further investigated the importance of balancing the pyruvate level via alcohol dehydrogenase in Arabidopsis alcohol dehydrogenase knock out mutants and concluded that alcohol fermentation plays an important role to prevent anoxia by controlling the level of pyruvate even under aerobic conditions. While the lower level of SUS is most likely a mechanism to control the pyruvate level to prevent anoxia, increasing levels of alcohol dehydrogenase (spots 5814, 5608, 5359, 5958) to metabolize pyruvate to ethanol is most likely a second important mechanism (Table 1, Table 2, Figure S8 in Supporting Information). This up regulation of the fermentative pathway could be a way to ensure the energy status of the cells under stress conditions while preventing anoxia. Zabalza and co-workers investigated the correlation between the induction of fermentation and the energy status (ratio of ATP to ADP).31 They concluded that the fermentative enzymes were induced via changes in the energy status caused by a decrease in oxygen. Nongreen tissues, like our samples, are entirely dependent on glycolysis and oxidative phosphorylation for their ATP production.

ATP production is important. We observed not only that from 4 days on the ATP-producing alcohol dehydrogenase reaches its highest level in the sucrose treatment (Table 2) and has its highest level in the sorbitol treatment (Table 3) but also that the ATP-producing phosphoglycerate kinase (2903) has at 4 days the highest level in the sucrose treatment and the lowest level in the control treatment (Table 1). In the sucrose treatment, the level of phosphoglycerate kinase increases continuously to reach its highest level at 14 days (spots 2903, 3935, 4550, 4969, Table 2).

As can be concluded from Table 1 and Table 3, the induction of the fermentation to produce ATP is not specific only for the sucrose treatment. Analogously as in the sucrose treatment, osmotic stress and the uptake of sorbitol and sucrose consume ATP and induce initially a higher sucrose metabolism followed by a burst in respiration, which leads to oxygen depletion. This leads to a shortage of ATP, which induces a switch toward a higher fermentation. However, in contrast to the sucrose treatment, sucrose might be limited in the sorbitol treatment. This might be reflected in a survival drop after 4 days and the highest abundance level of both enzymes responsible for the fermentation of pyruvate (pyruvate decarboxylase (5308, 5615) and alcohol dehydrogenase (5608, 5359) at the optimal acclimation time (4 days) (Table 3). The abundance level of both enzymes decreases after 4 days in the sorbitol treatment most likely because of the limited availability of sucrose and its breakdown products. This is an indication that ATP production plays an important role during this acclimation and might additionally explain the difference in acclimation efficiency between the sorbitol and sucrose treatment. Sucrose is not only a good compatible solute protecting membrane structures and proteins during dehydration, it is moreover, in contrast to sorbitol, an important energy source.

Interaction SUS-UGPase. In our previous work,11 it was hypothesized that during the sucrose acclimation treatment UGPase and SUS interact antagonistically. In this study, we present kinetic data that confirm this theory. The PCA analysis shows that different SUS isoforms contribute similarly to PC1 (data not shown). The individual univariate statistics (ANOVA and multiple comparison test) prove for UGPase (spot 3865) that its abundance at 14 days is significantly higher than at 8, 4, and 2 days (Table 2). Phosphoglucomutase (4415) shows a similar change over time as UGPase. Comparison of both abundance patterns of SUS and UGPase/phosphoglucomutase confirms the opposite expression pattern (Figure S9 in Supporting Information, Table 2). This is probably a mechanism where UDP-glucose production is assured via the conversion of glucose-6-P after that the SUS producing pathway is downregulated for a longer time. The higher level of UGPase and phosphoglucomutase is only achieved a few days after SUS is present in lower amounts.

Pentose Phosphate Pathway. Sharing common metabolites with the glycolytic pathway, the pentose phosphate pathway can metabolize sugars in plant cells providing the cell with reductive power and a number of sugar phosphates. The pentose phosphate pathway is directed by the initial reaction catalyzed by glucose-6-P-dehydrogenase, which is controlled by the ratio of NADPH to NADP+ (inhibition by NADPH).32 NADPH is thought to drive reductive steps associated with various biosynthetic reactions occurring in the cytosol. In nongreen tissues such as meristems, the pentose phosphate pathway may also supply NADPH for biosynthetic reactions such as lipid biosynthesis and nitrogen assimilation. The main sinks for NADPH are ROS signaling and metabolism and processes such as folate turnover and fatty acid biosynthesis.33 NADPH is also involved in regenerating low-molecular-mass antioxidants in several of the ROS-detoxification pathways. Scharte and co-workers have shown that tobacco plants were more tolerant toward biotic and abiotic stress (drought) after the introduction of an engineered NADPH tolerant glucose-6P-dehydrogenase isoform.32 This resulted in an exceptionally high NADPH production by preventing the feedback of NADPH. We observed during the sorbitol treatment that several enzymes of the pentose phosphate pathway (glucose-6-P dehydrogenase (5606), 6-phosphogluconate dehydrogenase (5354, 5635), and transketolase (4410)) have their highest abundance at the optimal acclimation time (4 days) (Table 3) and are correlated in the PCA analysis. This is an indication that NADPH production might play an important role during osmotic acclimation and the decrease after 4 days might again be correlated to the depletion of sucrose in the sorbitol treatment and the drop in regeneration.

Defense and Stress. Our data show also that proteins involved in stress and defense play a role in acclimation: 1-aminocyclopropane-1-carboxylate (ACC) oxidase (2715, Table
S1 in Supporting Information), ABA [abscisic acid] -, stress-, and ripening-induced protein (ASR) (5084, 5128, 5347, Table S1 and Table S3 in Supporting Information), ascorbate peroxidase (3162, Table S1 in Supporting Information), monodehydroascorbate reductase (3102 Table S1 in Supporting Information, 4753 Table S2 in Supporting Information), glutathion transferase (GST) (4825, Tables S1, S2, S3 in Supporting Information), heat shock proteins HSP70 (Table S1 in Supporting Information) and lipoxygenase (4387, Table S1 in Supporting Information). Since the focus of this paper is on the glycolysis, we will not discuss this further.

**General Conclusions**

We show that during acclimation of our meristems a switch takes place toward a higher fermentation for ATP production. This could be an essential mechanism to control the respiration rate and might be a way to ensure the energy status of the cells under stress conditions while preventing anoxia and high ROS overproduction. From the sorbitol time series, we hypothesize that the optimal duration of acclimation treatment is associated with the highest intracellular concentration of compatible solute (sucrose/sorbitol), a high energy status, and a high reducing power. The reason why a sucrose treatment is more powerful than a sorbitol treatment is that sucrose is the key metabolite to produce ATP and reducing power via glycolysis and pentose phosphate shunt. The amount of sucrose is, contrary to the sucrose treatment, limited in the sorbitol treatment, and the regeneration goes down when the availability of sucrose and hence the generation of ATP and reducing power drops. Additionally, our results point also toward proteins generally involved in stress and defense.

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**Supporting Information Available:** This material is available free of charge via the Internet at http://pubs.acs.org.

**References**


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