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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*[4] 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

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dehydration treatment associated with cryopreservation.[6] Sucrose can be considered as a special osmotic component because it is the major photosynthetic end-product and translocated carbohydrate. Moreover, in most plants sucrose (and its constituents glucose and fructose) have been recognized as important signaling molecules that affect a variety of physiological responses and many jasmonate-, ABA-, and stress-inducible genes are (co)regulated by sugars.[1,7−10]

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 unravel 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.

Marerials and Methods

Plant Material. *In vitro* plants were provided by the Bioversity 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.[6,11] Multiple shoot meristem cultures were initiated and maintained as described by Strosse and co-workers.[12] The meristem cultures were kept on three 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 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.

Cryopreservation and evaluation of the regeneration was executed as described by Panis and co-workers.[5] Briefly, acclimated meristems are subjected to a severe dehydration prior to freezing in liquid nitrogen. A high water content prior 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 meristems survived 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.[13] Proteins were labeled with Cy2, Cy3, and Cy5 (GEHealthcare, Diegem, Belgium) and scanned as described by Carpentier and co-workers.[14]

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 Carpentier and co-workers.[15]

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

Protein Identification and EST Annotation. Protein identification is based on peptide homology analysis of mass spectrometry derived peptide sequences. The sequence from protein isolation to protein identification is essentially performed as described previously.[11] Significant EST hits were further analyzed for annotation using the PBLAST interface. Identifications obtained by the NCBI Viridiplantae 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 server (http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/netblast.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_r. The number of transmembrane domains was calculated by the TMHMM Server v 2.0 (http://protfun.net/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.invitrogen.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 Construction 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 PCR Kit (Clontech Laboratories, www.clontech.com) were also used in the preparation of the cDNA library. The 5′-ends of the cDNA clones were sequenced at the J. Craig Venter Institute in Rockville, MD (http://www.jcvi.org/cms/home/).

A total of 11,070 ESTs were generated and stored at the Musa ESTs database, DATAMusa (http://genoma.embrapa.br/musa/pt/DATA_musa.html) and submitted to NCBI (FL657457.1 to FL668268.1). The raw data were analyzed using the EGassembler web server (http://egassembler.hgc.jp/), which provides an automated as well as a user-customized analysis tool for cleaning, repeat masking, vector trimming, organelle masking, clustering, and assembling the ESTs and genomic fragments.[16] In total, the ESTs encompassed 1,418,992 bp of high quality sequence.

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 ($\alpha \leq 0.01$) showed significant differences among the acclimation treatments 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, $\alpha \leq 0.05$,) and there was moreover a strong indication that the osmotic treatment with sucrose is more powerful than the sorbitol treatment (Figure 1).

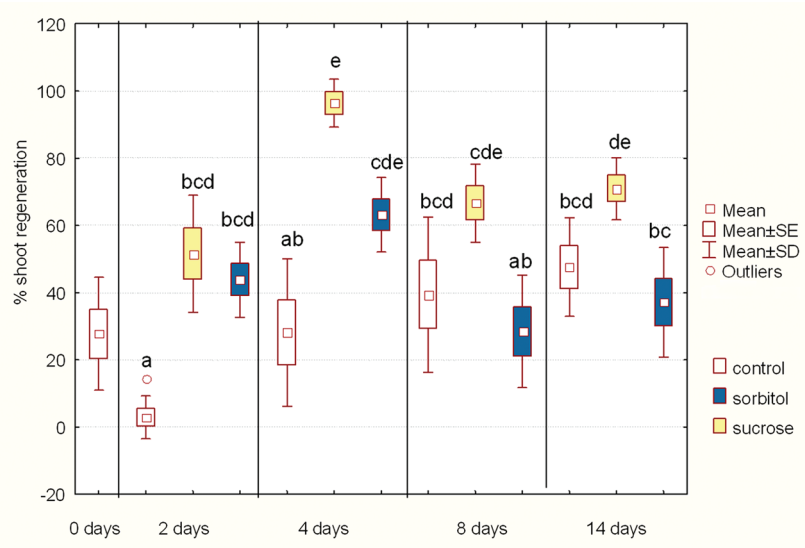

Figure 1. Acclimation experiment, an in vitro setup. Banana meristems were subjected to a control, sorbitol, and sucrose acclimation treatment for 2, 4, 8, and 14 days, and the percent of shoot regeneration was determined after dehydration and freezing. Differences were evaluated by two-way analysis 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.

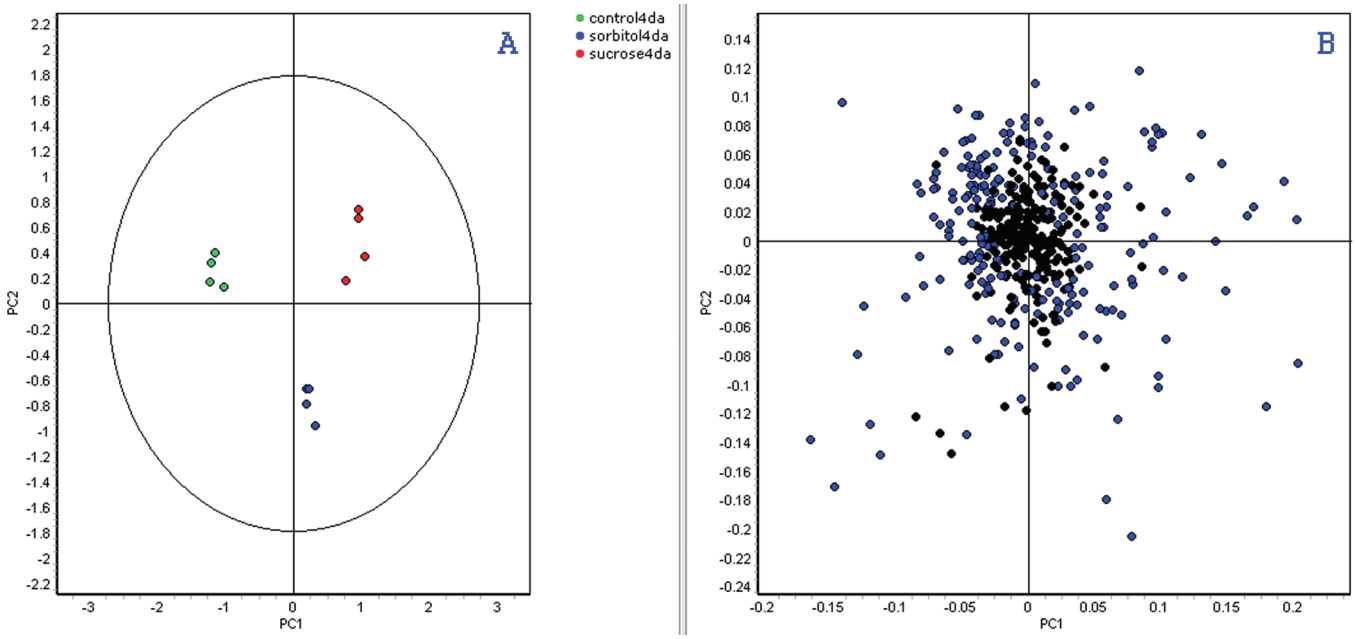

Figure 2. 2D DIGE experiment multivariate analysis. (A) Score plot. (B) Loading plot (proteins significantly different by ANOVA FDR are indicated in blue). Via the score plot and loading plot, proteins can specifically be correlated to the sucrose acclimation treatment (first (upper right) quadrant and 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.

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 2 A). 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 ($\alpha \leq 0.05$). PCA and ANOVA are two important ways to characterize the proteome.[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

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

				multiple comparison[a] treatment		
ID no.	annotation via the closest homologue	PCA classification	ANOVA (FDR)	suc	sorb	cont
5814	alcohol dehydrogenase	osmotic stress high	0.0000	a	a	b
5608	alcohol dehydrogenase	osmotic stress high	0.0000	a	a	b
5359	alcohol dehydrogenase	osmotic stress high	0.0001	a	a	b
484	phosphoenolpyruvate carboxylase	osmotic stress high	0.0059	a	a	b
5158	SUS	osmotic stress high	0.0007	b	a	c
3319	enolase	sucrose high	0.0000	a	c	b
3126	enolase	sucrose high	0.0008	a	b	b
4439	enolase	sucrose high	0.0094	a	b	b
4956	phosphoenolpyruvate carboxylase	sucrose high	0.0043	a	b	b
2903	phosphoglycerate kinase	sucrose high	0.0001	a	b	c
4556	phosphoglyceromutase	sucrose high	0.0004	a	c	b
5615	pyruvate decarboxylase	sucrose high	0.0043	a	ab	b
4922	SUS	sucrose high	0.0001	a	b	a
5043	SUS	sucrose high	0.0009	a	b	b
5090	SUS	sucrose low	0.0000	b	a	a
4703	SUS	sucrose low	0.0001	c	b	a
4834	SUS	sucrose low	0.0001	c	b	a
5232	SUS	sucrose low	0.0001	b	a	a
5461	SUS	sucrose low	0.0003	b	a	a
4989	SUS	sucrose low	0.0055	b	a	ab
4578	SUS	sucrose low	0.0116	b	a	a
4845	succinate dehydrogenase	osmotic stress low	0.0015	a	b	a
5480	SUS	osmotic stress low	0.0001	b	c	a

[a] suc = sucrose treatment, sorb = sorbitol treatment, cont = control treatment. Significance: $\alpha \leq 0.05$. Abundance: a > b > c; results with the same letter indicate no significant difference in abundance.

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

				multiple comparison (days)[a]			
ID no.	annotation via the closest homologue	PCA classification	ANOVA (FDR)	2	4	8	14
5615	pyruvate decarboxylase	early phase	0.0048	a	a	a	b
3372	pyruvate orthophosphate dikinase	early phase	0.0013	a	a	b	c
4703	SUS	early phase	0.0029	a	b	c	c
5461	SUS	early phase	0.0112	a	b	b	b
5232	SUS	early phase	0.0228	a	b	b	b
5090	SUS	early phase	0.0283	a	b	b	b
5043	SUS	early phase	0.0330	ab	a	a	b
4834	SUS	early phase	0.0493	a	ab	b	b
4578	SUS	early phase	0.0497	a	ab	b	b
5958	alcohol dehydrogenase	late phase	0.0046	b	a	a	a
4415	phosphoglucomutase	late phase	0.0067	c	bc	b	a
4969	phosphoglycerate kinase	late phase	0.0083	c	b	b	a
4540	phosphoglycerate kinase	late phase	0.0133	c	ab	b	a
2903	phosphoglycerate kinase	late phase	0.0138	b	b	a	a
3935	phosphoglycerate kinase	late phase	0.0221	c	bc	ab	a
4556	phosphoglyceromutase	late phase	0.0063	c	b	b	a
3782	phosphoglyceromutase	late phase	0.0067	b	b	a	a
4922	SUS	late phase	0.0365	b	a	ab	b
4529	triose phosphate isomerase	late phase	0.0035	c	b	a	a
3865	UGPAse	late phase	0.0044	b	b	b	a

[a] Significance $\alpha \leq 0.05$. Abundance: a > b > c; results with the same letter indicate no significant difference in abundance.

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 ($\alpha \leq 0.05$). 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

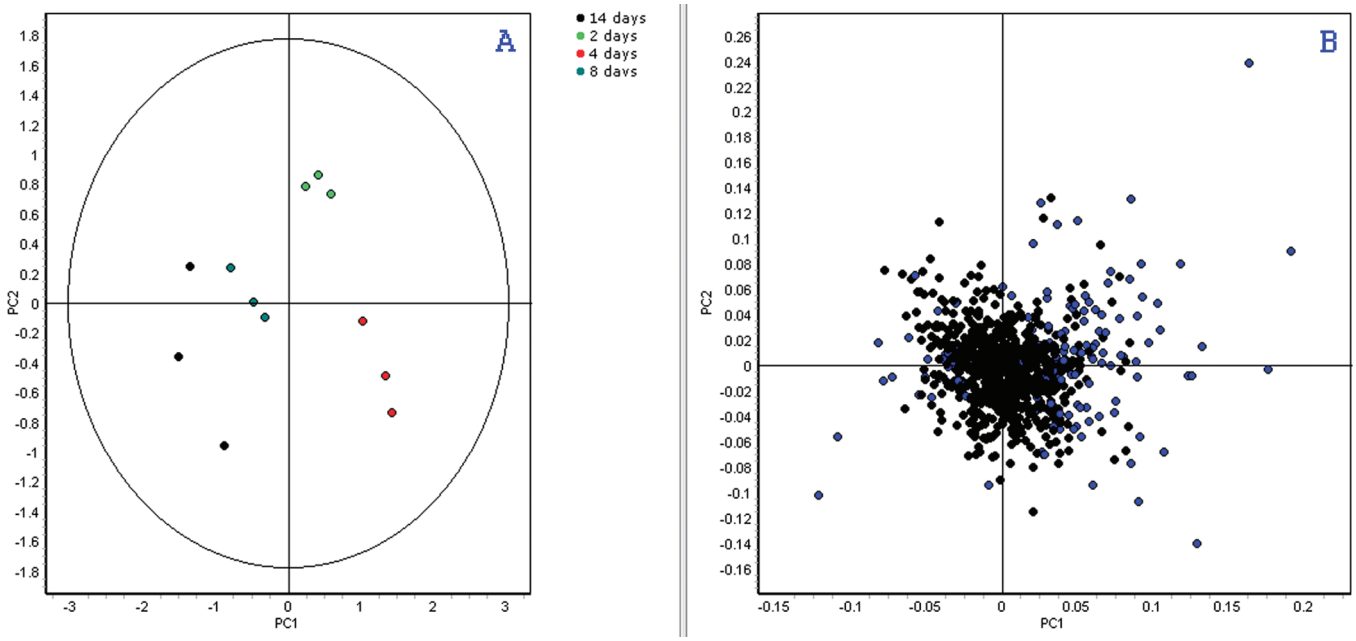

Figure 3. 2D DIGE experiment at 2, 4, 8, and 14 days of sorbitol acclimation treatment, a multivariate analysis. (A) Score plot. (B) Loading plot (proteins significantly different by ANOVA FDR are indicated in blue). PC1, explaining 43.7% of the variance, can be correlated to time. The two extreme time points are 4 and 14 days.

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

ID no.	annotation via the closest homologue	PCA classification	ANOVA (FDR)	multiple comparison (days)[a]			
				2	4	8	14
5354	**6-phosphogluconate dehydrogenase**	**4 days high**	**0.0007**	**b**	**a**	**c**	**c**
5635	**6-phosphogluconate dehydrogenase**	**4 days high**	**0.0035**	**a**	**a**	**b**	**b**
4533	**aldo/keto reductase**	**4 days high**	**0.0112**	**b**	**a**	**b**	**b**
5606	**glu-6-phosphate 1-dehydrogenase**	**4 days high**	**0.0220**	**b**	**a**	**b**	**b**
4410	**transketolase-like protein**	**early phase**	**0.0009**	**a**	**a**	**b**	**b**
5608	alcohol dehydrogenase	4 days high	0.0003	b	a	c	c
5359	alcohol dehydrogenase	4 days high	0.0076	b	a	b	b
5353	aldehyde dehydrogenase	4 days high	0.0224	b	a	b	b
4441	NADP-dependent malic enzyme	4 days high	0.0012	b	a	c	d
5308	pyruvate decarboxylase	4 days high	0.0277	ab	a	b	b
5615	pyruvate decarboxylase	early phase	0.0003	a	a	b	c
3178	pyruvate dikinase	early phase	0.0319	a	b	b	b
3782	phosphoglycerate mutase	late phase	0.0346	c	bc	ab	a
4578	SUS	late phase	0.0414	bc	c	ab	a

[a] Significance $\alpha \leq 0.05$. Abundance: a > b > c > d; results with the same letter indicate no significant difference in abundance.

variance. 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 ($\alpha \leq 0.05$). 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*I* and M_r for both methods. The EST libraries cover genes coding for proteins with a broad p*I* and M_r range (respectively, 4−13 and 1000−234000), while the 2DE approach is restricted to the chosen p*I* (4−7) and proteins that are too big or too small are not quantified (range 11000−171000)

(Figure S4 in Supporting Information). The 2DE also does not cover proteins that are too hydrophobic (we found up to 1 transmembrane helix while the EST library contains transcripts coding for proteins with up to 14 transmembrane helices) (Figure S5 in Supporting Information).

Discussion

Acclimation. Our experimental setup based on the use of meristem cultures proved to be an attractive approach to evaluate osmotic acclimation and to study tolerance toward a severe dehydration in meristems. In previous reports, we tested a wide range of varieties all differing in response and in acclimation capacities[17] and investigated the proteome of the two most extreme phenotypes.[11] To gain insight into the different aspects of the acclimation process and the role of sucrose, we focused here on the most tolerant variety and followed its proteome over time. In order to dissect the different aspects of the sucrose-mediated acclimation, we included, in addition to the high sucrose and a control treatment, the sugar alcohol sorbitol in the experimental design at the same molarity to create a similar osmotic effect. Sugar alcohols are widely used in osmotic stress studies as poorly metabolizable compatible solute and as osmotic control.[9,18] Because sorbitol is in some species the primary photosynthetic product,[19] we first checked whether sorbitol can be used as carbon source by the banana meristem cells. Sorbitol (0.09 M) does not support the growth of banana meristems cells (results not shown). An osmotic treatment with 0.4 M sucrose enhances specifically the survival rate of our tolerant variety.

Sucrose Synthase, the Main Sucrose-Metabolizing Enzyme. Sucrose synthase (SUS) catalyzes the reversible conversion of sucrose into fructose and UDP-glucose. A full understanding of this complex gene family is not yet acquired. Moreover, it has been found that there is a poor correlation between SUS mRNA and protein levels[20] and that post-translational modifications play a role.[3,21,22]

In this report, we provide evidence that SUS is the dominant enzyme in *in vitro* meristems and that post-translational modifications (PTM) complicate the interpretation. Seventeen different spots were identified as SUS (Table S4 in Supporting Information: 4578, 4703, 4834, 4922, 4989, 5043, 5046, 5090, 5158, 5232, 5314, 5400, 5461, 5480, 5548, 5742, 5890) but only 4 different mRNAs were detected in the EST library (data not shown).

SUS activity can be regulated at the enzyme level by rapid changes in subcellular localization, phosphorylation, and carefully modulated protein turnover,[23] and it is known that SUS activity is subject to feedback inhibition by fructose.[24,25] Using the DIGE approach, we found that 7 isoforms were, compared to the control treatment, significantly less abundant after the sucrose treatment (Table 1: 4578, 4703, 4834, 5090, 5232, 5461, 5480). This might point toward a feedback mechanism by sucrose or one of the metabolites to down regulate transcription and/or to modify SUS post-translationally and/or to enhance its proteolytic turnover under high sucrose concentrations. A post-translational modification might explain why we observe next to a lower abundance of 7 isoforms also a significantly higher abundance of 2 isoforms (Table 1: 5043, 5158); this might be a shift from one isoform into the other. It is known that a first phosphorylation at S^{15} activates SUS, while a second phosphorylation at S^{170} targets the protein for ubiquitin-mediated degradation.[21] The higher abundance of those spots might be the S^{170} phosphorylated isoforms targeted for breakdown. The analysis in time of the sucrose treatment confirmed that the abundance of 6 of the 7 isoforms (Table 2: 4578, 4703, 4834, 5090, 5232, 5461; Figure S6 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,[2] 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[11,17] 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 exterior 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.[23] The effect of low internal oxygen concentrations has been investigated by Bologa and co-workers.[26] 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.[27]

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 glycolitic 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-6P-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.

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