Identification of drought-responsive compounds in potato through a combined transcriptomic and targeted metabolite approach

Danièle Evers1,*, Isabelle Lefèvre1, Sylvain Legay1, Didier Lamoureux1, Jean-François Hausman1, Raymundo Oscar Gutierrez Rosales2, Luz Rosalina Tincopa Marca2, Lucien Hoffmann1, Merideth Bonierbale2 and Roland Schafleitner2

1 Centre de Recherche Public - Gabriel Lippmann, Department EVA, 41, rue du Brill, L-4422 Belvaux, Luxembourg
2 International Potato Center, Germplasm Enhancement and Crop Improvement Division, La Molina 1895, Apartado 1558, La Molina, Lima 12, Peru

* To whom correspondence should be addressed: evers@lippmann.lu

Received 7 December 2009; Revised 15 January 2010; Accepted 24 February 2010

Abstract

Two potato clones (Solanum tuberosum L.) of the Andean cultivar group, called Sullu and SS2613, with different drought-tolerance phenotypes were exposed to a continuously increasing drought stress in a field trial. At the physiological level, while relative leaf water contents were similar in both clones, osmotic potential was lower in Sullu and declined more strongly during drought compared with SS2613. In the drought-stressed plants, tuber yield was reduced by about 70% compared with control plants in both clones. Potato cDNA microarrays and target metabolite analysis were performed on leaves sampled at several time-points after the onset of drought. At the transcriptomic level, photosynthesis-related genes were already strongly repressed in Sullu after 28 d of withholding irrigation and even more strongly after a longer stress duration, whereas, in SS2613, repression occurred only after 49 d of soil drying; similarly, a strong perturbation of carbohydrate-related genes was observed in Sullu. At the metabolite level, differential accumulation of osmotically active solutes was observed between the two cultivars; indeed, in Sullu, contents of galactose, inositol, galactinol, proline, and proline analogues were higher upon drought stress compared with SS2613. These results point to different drought responses in the cultivars at the leaf level, with, however, similar tuber yield reductions. The previously shown tolerant clone Sullu lost part of its tolerance under the experimental conditions used here; it was, however, able to maintain an absolute yield three times higher than SS2613.

Key words: Abiotic stress, drought, microarrays, potato, polyamines, proline.

Introduction

Potato is the fourth most important crop in the world. It is generally considered to be drought-sensitive and this limits its production to areas with adequate rainfall or irrigation possibilities, the latter influencing the production price. The context of changing climatic conditions resulting in higher temperatures, dry periods, and more erratic rainfall patterns in many regions will increasingly affect potato production in many areas of the world. The identification of drought-tolerance traits and genes for potato would facilitate breeding for yield stability under water-limiting conditions. The drought sensitivity of potato impacts at virtually all stages of the crop, from emergence to tuber initiation and bulking (Mould and Rutherfoord, 1980). Drought tolerance in plants is intricately linked to photosynthesis and carbohydrate metabolism (Basu et al., 1999). An important plant response to low water availability is stomatal closure, which limits CO2 availability for photosynthesis, leading to an increase in photorespiration and a reduction in net photosynthesis. Moreover, under stress, reactive oxygen species (ROS) production is enhanced, resulting in DNA damage.
and lipid peroxidation (reviewed by Cruz de Cavalho, 2008). Genes implicated in the detoxification of ROS are found to be up-regulated in potato upon drought stress (Watkinson et al., 2008). Whether this is a direct response to drought stress or a secondary response to the oxidative stress is difficult to define.

Other drought stress-induced gene products are predicted to protect cellular structures. The late-embryogenesis-abundant (LEA) genes have been shown to be expressed in vegetative tissues during periods of water deficit (Bray, 1993; Valliyodan and Nguyen, 2006). Some of them, such as the dehydrins, have been found to act as chaperones that stabilize proteins and membrane structure in stressed plants. Another important response to drought is osmotic adjustment, that aims to maintain cell turgor during water deficit. The osmotic potential inside the cell is lowered by the accumulation of osmolytes in the cytoplasm (reviewed by Morgan, 1984). Genes coding for enzymes that are responsible for the accumulation of these osmolytes, also called compatible solutes, are induced during drought stress in several plants. At the metabolite level, this adaptation response is reflected by the accumulation of several amino acids and sugars, for example.

Necessary for the drought stress response at the molecular level is the adaptation of transcription regulation to the stress situation. At the transcriptional level, out of at least four independent regulatory systems for gene expression in response to water stress in Arabidopsis thaliana, two are abscisic acid (ABA)-dependent and two are ABA-independent (Valliyodan and Nguyen, 2006). Some important transcriptional regulators are the MYC and MYB proteins that function as activators in one of the ABA-dependent regulatory systems (Valliyodan and Nguyen, 2006). In addition, the dehydration-responsive element (DRE) is also involved in the ABA-independent regulatory systems.

While studies on the short-term response to drought stress provide much information on stress perception and differential activation of signalling pathways, gene expression changes have been analysed on potatoes exposed to a prolonged period of drought stress and information has been gathered on the adaptation responses to lasting water deficit. Understanding these adaptation mechanisms is vital to identify traits that improve stress tolerance in crops through breeding and transgenic strategies. Thus, two potato clones (Solanum tuberosum subsp. andigenum) with different drought-tolerance phenotypes were exposed to drought stress in a field trial. One of them, Sullu, was previously shown to be tolerant under drought stress (Schafleitner et al., 2007), while the other cultivar, SS2613, was susceptible. The present work aims to identify putative drought tolerance markers through a transcriptomic approach followed by a targeted metabolite analysis. To this end, gene expression in both clones was studied at two time points after drought onset and the results were analysed using the MapMan tool (Usadel et al., 2005) focusing mainly on the expression changes of genes involved in photosynthesis, carbon and amino acid metabolism as well as on biochemical modifications related to the latter.

**Materials and methods**

**Plant material and culture conditions**

Sprouted seed tubers of the Andean potato clones Sullu and SS2613, both *S. tuberosum* subsp. *andigenum*, were planted together with a further 25 Andean potato clones in blocks of five plants in a random complete block design with four replicates on hunic soil (Mollisol, pH 4.0, light salinity (2-4 dS m⁻¹), high organic matter (>30%), normal cationic relations) in four rain shelters at the CIP experimental station Huancayo, Peru, at 3200 masl, on 9 October 2006. Each rain shelter was equipped with a plastic roof and plastic barriers 60 cm below ground to prevent uncontrolled water inflow and with plastic nets to exclude insects. The plots were fertilized with 100:160:120 kg ha⁻¹ nitrogen:phosphorus:potassium before planting and with 100 kg ha⁻¹ nitrogen at hillling, 28 d after planting. Fungicide and insecticide sprays (Mancoceb, Propineb, Dimetoen, Cymoxanil, Permetrin, and Cypermetrin) were applied in weeks 4, 6, 8, and 10 after planting according to the suppliers’ recommendations. Plants were watered by drip irrigation and soil water potential was kept between 0 and −0.02 MPa. In the drought plot, irrigation was stopped on day 86 after planting and drought was applied for 58 days (144 d after planting), while the control plot was continuously irrigated. After the drought period, irrigation was resumed and continued until day 156 after planting. On day 163, the haulms were cut and tuber harvest took place on day 170 after planting.

Soil water content was determined gravimetrically (g of water per g of soil) for a soil profile from 0 cm to 50 cm depth, on days 0, 28, 37, 49, 53, and 58 after drought (AD) onset in each replicate plot and treatment.

Relative water content was determined on 28 d and 49 d AD in the third fully expanded leaf. Leaves were excised, weighed (FW), floated on distilled water for at least 8 h for full hydration of the plant tissue, blotted dry, and weighed again (TW). Dry weight (DW) was subsequently determined after oven-drying for 2 d. Relative water content (RWC) was calculated as RWC=−(FW–DW)/(TW–DW)×100.

Osmotic potential was measured with a dewpoint microvoltmeter HR-33T (Wescor Inc.) in a psychrometric chamber (Model C-52; Wescor Inc.) after calibration with 100, 290, and 1000 mmol kg⁻¹ NaCl standards.

**Yield analysis**

Tubers of the three central plants of a block were harvested 170 d after planting. Fresh tuber weight per plant was determined immediately after harvest. An aliquot of the tubers was oven-dried for dry-mass determination.

**Sampling for gene expression and metabolite analysis**

Third fully-expanded leaves were harvested and pooled from five plants per block on days 15, 28, and 49 after drought onset. Three replicate blocks were sampled for each clone for metabolite and gene expression analysis. The harvested leaves were partitioned into two aliquots and were shock-frozen in liquid nitrogen and kept at −80 °C until RNA isolation or lyophilization for metabolite analysis.

**RNA extraction, microarray hybridization, and analysis**

RNA samples were isolated from leaves using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the recommendations of the supplier. Samples were cleared from Trizol and other interfering substances using the RNeasy cleaning plant mini kit from Qiagen (Leusden, The Netherlands). A DNase treatment (Qiagen) was also applied to remove any residual genomic DNA.

Microarray analysis was performed on leaves sampled 28 d and 49 d AD. Labelling of 20 µg total RNA per sample was performed following the protocol as described by Legay et al. (2009) using Alexa dyes instead of cyanine dyes. Two biological replicates were
performed for each time-point, using a different leaf as starting material for the extraction. Cy5-labelled cDNAs of drought-stressed plants were hybridized versus Cy3-labelled cDNAs of control plants. Each slide was replicated with a dye-swap (inverted labelling). Data acquisition was performed according to Legay et al. (2009).

Statistical analysis

To analyse files generated by Genepix® Pro 6.0 software, data were imported into Acuity 4.0 software (Molecular Devices Corporation, Union City, CA, USA) and normalized with the print-tip Lowess method. The fold-change between stress and control conditions was expressed by the following formula: log ratio (stress/control)=log2((signal median 635–background median 635)/(signal median 532–background median 532)).

For a first overview of global response to drought stress, hierarchical clustering (according to Bray-Curtis) was performed on ANOVA-selected clones with a stringent P value <0.0005 to retain only the most significant genes.

Secondly, a ‘one sample t test’ was performed for the four slides of each time-point using the formula below:

\[
t = \frac{\bar{x} - \mu_0}{s/n}
\]

where \(\bar{x}\) is the mean of the four replicates of the spot, \(\mu_0\) is the mean of the population (in our case \(\mu_0=1\) in linear scale or \(\mu_0=0\) in log scale), \(s\) is the standard deviation of the four replicates and, finally, \(n\) is the sample size. Probes with a \(P\) value <0.05 and a log ratio below –0.5 and above 0.5 were selected for further analysis in MapMan (Usadel et al., 2005).

Quantitative RT-PCR analyses

Transcription of total RNA was performed using TaqMan® Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions using random hexamers. Primers were designed with primer express 2.0 (Applied Biosystems) with the following final concentrations in 25 μl final volume: MasterMix at 1 volume, forward and reverse primers at 300 nM, M dNTP Mix, 2.5 μM random hexamers, 1.25 U RnaseI, 1.25 U M. l 5′-acetyl DL-proline was added as internal standard (IS) to the injection standard, an external column heater (Bio-Rad, Hercules, CA, USA), an external column heater (Bio-Rad, Hercules, CA, USA), and a Finnigan MSQ quadrupole spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

Water-soluble carbohydrate and polyol extraction and measurement

Water-soluble carbohydrate and polyol extraction was performed following the method described by Oufir et al. (2008) on 100 mg (±10 mg) of freeze-dried leaves sampled 15, 28, and 49 d AD. Carbohydrate analysis was performed following the method described by Oufir et al. (2008). Trehalose, arabino, xylose, melibiose, stachyose, raffinose, maltose, verbascose, cellobiose, and cellotriose contents in the leaf extracts were below the detection limit (data not shown).

Proline, its analogues and betaines extraction and measurement

Extraction was performed following the method described by Oufir et al. (2009) on 60 mg (±10 mg) of freeze-dried leaves sampled 15, 28, and 49 d AD.

\(\text{N-acetyl tL-proline was added as internal standard (IS) to the extraction mixture at a final concentration of 100 \mu M in order to assess the recovery of the method. The injection standard, N-acetyl-L-galactosamine, was added immediately before the sample was injected through the chromatographic system at a final concentration of 20 \mu M.}

Measurement was performed following the method described by Oufir et al. (2009) with slight modifications. Isocratic HPLEC-MS experiments were performed on a Dionex BioLC chromatograph, with an ASS50 autosampler, a GS50 gradient pump (Dionex Corp., Sunnyvale, CA, USA), an external column heater (Bio-Rad, Hercules, CA, USA), and a Finnigan MSQ quadrupole spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase was degassed (vacuum filtration through a Millipore HA 0.45 mm filter) Milli-Q water containing 5 mg l\(^{-1}\) disodium calcium salt-EDTA.

Prior to initial use and after running about 50 samples, the column was regenerated by passing a 500 mg l\(^{-1}\) disodium calcium salt-EDTA solution at a flow rate of 0.1 ml min\(^{-1}\) overnight. The
column was then washed with the mobile phase until baseline stabilization.

The analytical column was an Aminex HPX-87C column (300×7.8 mm ID, 9 μm particle size) with a micro guard Carbo C (4.6×3 mm) cartridge (Bio-Rad, Hercules, CA, USA). The flow rate was 0.8 ml min⁻¹ and the column was heated at 40 °C.

MS data were acquired on a quadrupole spectrometer with an electrospray interface (ESI). The probe temperature was fixed at 500 °C with a needle voltage of 3.5 kV. All chromatographic and MS data were interpreted using the softwares Chromeleon 6.5 and Xcalibur 1.3.

All compounds of interest were quantified using seven-point calibration curves with custom-made external standard solutions, ranging from 0.625 to 20 μmol l⁻¹; every 10 injections, a check standard solution was used to confirm the calibration of the system.

**Soluble free polyamines extraction and measurement:** Free polyamines (PAs) were extracted according to Oufr et al. (2008) on 70 mg (±10 mg) of freeze-dried leaves sampled 15, 28, and 49 d after drought.

For fluorescence detection, PAs were derivatized by dansylation according to Smith and Davies (1985) with some slight modifications. Two hundred μl aliquots of the supernatant were added to 200 μl of saturated sodium carbonate and 500 μl of dansyl chloride in acetonitrile (20 mg ml⁻¹) and vortexed for 10 s. The mixture was incubated at 60 °C for 1 h in the dark. To remove the excess dansyl chloride, 250 μl of proline (150 mg ml⁻¹) were added. After 1 h of incubation in the dark, acetonitrile was evaporated under nitrogen. The PAs were then extracted with 1 ml ethyl acetate with vigorous vortexing for 60 s. The mixture was stored for 15 min at 4 °C in order to obtain a good separation between organic and aqueous phase. The upper organic phase was collected and dried in a vacuum concentrator (Heto, Thermo Electron Corporation, Waltham, MA, USA). The dried extract was dissolved in 1 ml of methanol, filtered through 0.45 μm Aerodisc GHP syringe filter and 10 μl of sample were injected on a Nucleodur C18 Pyramid column (125×4.6 mm ID; 3 μm particle size) (Macherey-Nagel, Düren, Germany) maintained at 40 °C.

HPLC-FLD (High performance liquid chromatography coupled with a fluorescence detector) analyses were performed on a Dionex BioLC chromatograph, with an AS50 autosampler, a GS50 gradient pump (Dionex Corp., Sunnyvale, CA, USA) and a Dionex RF 2000 Fluorescence Detector (Dionex, Sunnyvale, CA) with the excitation wavelength at 340 nm and the emission wavelength at 510 nm. The flow of the mobile phase was 1.0 ml min⁻¹. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The gradient programme was as follows: 40% B to 91% B (20 min), 91% B to 100% B (2 min), 100% B (3 min), 100% B to 40% B (1 min) and column equilibration at 40% B during 4 min. Total run time was 30 min.

Free PAs were quantified using five-points calibration curves with custom-made external standard solutions and internal standard (1,7-diaminoheptane-2HCl), ranging from 3.125 μmol l⁻¹ to 50 μmol l⁻¹ and every ten injections, a check standard solution was used to confirm the calibration of the system. The internal standard gave information about the recovery of the extraction and derivatization during the evaluation of PA content.

**Statistical analyses for biochemical compounds:** The data were analysed by two-ways ANOVA, with treatment and duration of treatment as main effects. Multiple comparison procedure between means was performed with a Tukey test with confidence limit of 95% (SigmaStat for Windows version 2.03).

**Results and discussion**

**Responses to drought stress at the physiological level**

On day 86 after planting, irrigation was stopped in the drought plot for 58 d in order to expose the plants to water stress during tuberization and bulking. Meanwhile, the control plot was irrigated continuously. Thirty-seven days after the onset of drought, soil water content reached values of around 53% in the control plot and 32% in the drought plot and decreased further to 29% by day 53 after drought (AD) and to 25% on day 58 AD (Fig. 1). Soil water levels were identical for both clones, Sullu and SS2613, as assessed by individual measurements. Relative leaf water contents were similar between control and stressed plants, as well as between genotypes (Table 1). Osmotic potential was generally lower in Sullu than in SS2613 and declined stronger during drought treatment in Sullu than in SS2613. In Sullu, under drought, osmotic potential reached –2.17 MPa (Table 1). The extremely low osmotic potential in Andean native potato clones under stress might contribute to turgor maintenance under strong water-stress conditions. These results indicate that both cultivars are able to keep the relative leaf water content constant despite a decrease in osmotic potential in Sullu. Decreased osmotic potential is considered a potential cellular mechanism of drought resistance.

**Table 1.** (a) Relative water content and (b) osmotic potential in Sullu and SS2613 exposed to drought or under control conditions.

![Fig. 1. Soil water content in drought and irrigated field plots from day 28 to day 58 after drought.](image)

<table>
<thead>
<tr>
<th></th>
<th>Sullu</th>
<th>SS2613</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drought</td>
<td>Control</td>
</tr>
<tr>
<td>Day 28</td>
<td>74% (±2%)</td>
<td>78% (±3%)</td>
</tr>
<tr>
<td>Day 49</td>
<td>71% (±2%)</td>
<td>74% (±2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sullu</th>
<th>SS2613</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drought</td>
<td>Control</td>
</tr>
<tr>
<td>Day 28</td>
<td>–1.71 (±0.44)</td>
<td>–1.65 (±0.31)</td>
</tr>
<tr>
<td>Day 49</td>
<td>–2.17 (±0.53)</td>
<td>–1.6 (±0.35)</td>
</tr>
</tbody>
</table>
resistance as it enables turgor maintenance and growth continuation (Sakthivelu et al., 2008).

The mean yield in the control condition was 678 g FW plant$^{-1}$ for Sullu whereas it was 221 g FW plant$^{-1}$ for SS2613 (Table 2). Under the present drought stress conditions, yield was reduced in both clones by about 67%, resulting in a mean yield of 223 g FW plant$^{-1}$ for Sullu and 74 g FW plant$^{-1}$ for SS2613. The harvest index (tuber DM (dry matter)/total biomass DM) was better in all treatments for Sullu than for SS2613 (Table 2) indicating that Sullu allocates relatively more carbon to the tubers. In the present field trial, elevated day temperatures (average day temperature, 33.9 °C; average night temperature, 11.8 °C) under the rain shelters could explain the low yield potential of SS2613 in control conditions. These yield reductions were different when compared with previous experiments (Schafleitner et al., 2007) where the yield loss under drought was 24% for Sullu and 70% for SS1613 (average day temperature, 17.4 °C; average night temperature, 15.6 °C).

Responses to drought stress at the gene expression and metabolite level

Gene expression was measured in control and drought-exposed leaves of SS2613 and Sullu using the TIGR 10 k microarray. Real-time RT-PCR assays on a subset of eight candidate genes were performed for validation. The

Table 2. Tuber yield (g plant$^{-1}$) in Sullu and SS2613 under control and drought stress
Harvest index is indicated as tuber DM/total biomass DM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Drought</th>
<th>Yield loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (g plant$^{-1}$)</td>
<td>Harvest index</td>
<td>Yield (g plant$^{-1}$)</td>
</tr>
<tr>
<td>Sullu</td>
<td>678</td>
<td>0.30</td>
<td>223</td>
</tr>
<tr>
<td>SS2613</td>
<td>221</td>
<td>0.32</td>
<td>74</td>
</tr>
</tbody>
</table>

Fig. 2. Real-time PCR expression results and correlation real-time (log$_2$ ratio drought/control from normalized expression level)/microarray (log$_2$ ratio drought/control) on the validated genes.
correlation between the real-time RT-PCR expression values and the microarray approach amounted to $R^2=0.89$ (Fig. 2).

Hierarchical clustering (Fig. 3) demonstrated that many of the genes had similar expression patterns in both clones (see clusters 5 and 14, for example); in some cases, the differences were quantitative in nature, such as in clusters 1, 2, and 3. On the other hand, other genes were differentially regulated between both clones, indicating differential drought responses. Thus, in clusters 4 and 16, at 28 d AD, the expression ratio drought/control was higher in Sullu compared with SS2613; the opposite trend was observed in cluster 8. Clusters 4 and 8 contain many transcription factors (see Supplementary Fig. S3 at JXB online). The results indicate that, under the present experimental conditions, duration of drought treatment had a stronger impact on gene regulation than the genotype, as approximately only one-third of the genes used in the clustering analysis were different between Sullu and SS2613 at 28 d AD, and even less at 49 d after drought.

To understand the functional significance of drought-induced gene expression data better, the MapMan software (Usadel et al., 2005) was used to group the 10 k microarray probes into functional groups or ‘bins’. While in most bins up- and down-regulated genes were represented, some bins contained predominantly either up- or down-regulated genes (Table 3; see Supplementary data S4 at JXB online). Hereafter, several bins, for which related biochemical data are also presented, will be discussed in detail.

**Photosynthesis: photosynthesis-related genes are strongly repressed in Sullu**

In the present field trial, drought stress led to strong repression of photosynthesis-related genes in Sullu (Fig. 4A). The light reaction, Calvin cycle, and chlorophyll biosynthesis gene expression was particularly strongly down-regulated. In contrast to Sullu, photosynthesis-related genes were instead induced in SS2613 28 d AD. Under prolonged stress (day 49 AD), in both clones, photosynthesis-related transcription was predominantly down-regulated. In Sullu as well as SS2613, genes involved in photorespiration were induced at 28 d AD (Fig. 4A). Induction of photorespiration-related genes had already been observed by Schaffelner et al. (2007) in a similar study. Wingler et al. (1999) also reported on increased photorespiration during drought stress in barley; photorespiratory enzymes exerted increased control on photosynthetic electron transport and CO$_2$ fixation. At both time points during drought stress, stronger at 28 d AD and in Sullu, the proton gradient regulation protein 5 (PGR5) was induced. This gene is generally activated under high light and limiting CO$_2$ and facilitates cyclic electron transport at PSI (DalCorso et al., 2008). Long et al. (2008) showed that increased PGR5 led to improved photoprotection and drought tolerance. Down-regulation of linear electron transport, to match the reduced requirement for electrons, further minimizes reactive oxygen production.

The expression pattern of a light-harvesting chlorophyll $a/b$ binding protein, as assessed by real-time RT-PCR, also indicated a statistically significant expression decrease (Fig. 2), especially for Sullu under drought. Van Heerden and Laurie (2008) suggest that the response of photosynthesis during drought stress has an influence on yield in sweet potato. Here, the yield reduction under drought as well as the photosynthetic failure were both important in Sullu, probably, as previously explained, also due to the relatively high day temperatures in the present experiment. It remains to be tested whether stomatal or non-stomatal limitations play a major role in yield loss of this crop under drought stress.

**Carbohydrate metabolism: strong perturbation of related genes in Sullu, but accumulation of galactose and inositol**

Carbohydrate biosynthesis gene expression followed the trend observed with photosynthesis genes: sucrose phosphate synthase (SPS) and starch synthase were up-regulated in SS2613 and down-regulated in Sullu at day 28 AD (Fig. 4B). Again, under prolonged stress, most genes of this group were repressed in both clones. Sucrose synthase (SS) transcription was induced in both clones under stress and fructose-1,6-bisphosphate synthase transcription was strongly repressed in Sullu. These expression patterns suggest heavy impairment of sucrose metabolism particularly in Sullu under drought stress. Glycolysis gene transcription was also more strongly repressed by drought stress in Sullu than in SS2613. Genes of the tricarboxylic acid cycle were mostly repressed under stress in both clones (Fig. 4B).

Under prolonged stress, UDP-glucose-4-epimerase was strongly induced in both clones (see Supplementary Table S4 at JXB online: cell wall-related genes). This enzyme contributes to the regulation of the monosaccharide pool available for pectin production (Reiter and Vanzin, 2001), but is also involved in galactinol, stachyose, and raffinose biosynthesis. Galactinol synthase was significantly more expressed in drought-stressed plants, with a more important response at 49 d AD compared with 28 d AD, and to a greater extent in SS2613 compared with Sullu (Fig. 2). This gene acts in the synthesis of galactinol from UDP-galactose and myo-inositol. In *A. thaliana*, galactinol synthase isoform expression increased during drought and overexpression increased drought tolerance (Taji et al., 2002). Both UDP-glucose-4-epimerase and galactinol synthase play a key role in raffinose family oligosaccharides (RFO) biosynthesis. Transgenic overexpression of UDP-glucose-4-epimerase in *A. thaliana* conferred tolerance to salt, drought, and freezing stress, probably by increasing galactose-derived sugar alcohol levels (Liu et al., 2007).

At the biochemical level, carbohydrate content (Table 4) did not change with time in the control, whatever the cultivar, except for galactose, which increased in Sullu. After 28 d of drought treatment, an increase in glucose and fructose was observed in both cultivars. No significant difference in carbohydrate content occurred after 49 d of drought treatment despite a down-regulation of some genes
Fig. 3. Hierarchical clustering of 589 genes differentially expressed upon drought exposure in clone SS2613 and Sullu, 28 d and 49 d after drought. $P < 0.0005$. 
involved in sucrose biosynthesis or the up-regulation of sucrose degradation as previously described in carbohydrate metabolism. Nevertheless, transport of these compounds in other plant parts could not be excluded, as these molecules may be involved in signalling pathways. Actually, sugars have been shown to modify transcription of many stress-related genes (Rolland et al., 2002; Price et al., 2004; Gupta and Kaur, 2005). Temporary modification of some carbohydrate contents in our experiment could be related to signalling mechanisms rather than to osmotic adjustment or to the accumulation of storage products.

Concerning polyols, inositol content (Fig. 5) increased rapidly, 28 d AD, and the increase was much more important in Sullu than in SS2613. The level of galactinol was significantly higher 28 d AD in Sullu, but this trend disappeared after 49 d of drought treatment. Several studies have indicated an increase in polyol content following drought stress (Popp and Smirnoff, 1995; Patonnier et al., 1999; Taji et al., 2002). Polyols have been reported to be osmotically active solutes in response to abiotic stress. Their hydroxyl groups could effectively replace water in establishing hydrogen bonds in the case of limited water availability and therefore protect enzyme activities and membranes (Chaves et al., 2003).

Thus, concerning carbohydrate metabolism, biochemical changes do not clearly reflect gene expression changes. Similar observations have been reported previously (Renaut et al., 2009) and they might be due to translational and/or post-translational modifications. However, galactose, inositol, and galactinol contents were actually higher in drought-stressed Sullu plants, indicating increased levels of osmotically active solutes in this cultivar.

Nitrogen, sulphur, amino acid, polyamine, and secondary metabolism: proline and proline anlage contents increase in Sullu

Drought stress induced nitrite reductase and a chloroplast PII nitrogen-sensing protein, known to activate glutamine synthase (Chen et al., 2006), in both clones on day 28 AD (see Supplementary Table S4 at JXB online: N- and S-assimilation). Moreover, cysteine biosynthesis genes were up-regulated in both clones, together with sulphur uptake genes such as adenosine phosphosulphate reductase. Induction of those genes was reversed to repression under prolonged drought. Increased amounts of cysteine might serve glutathione biosynthesis. A proline biosynthesis gene (Δ1-pyrroline-5-carboxylate synthetase, P5CS) was up-regulated at 28 d AD in both clones (Fig. 2) and prolyl-4-hydroxylyase was induced in SS2613 late under drought conditions. Transgenic tobacco plants overexpressing P5CS were shown to have increased proline production which has been shown to be related to drought and salt stress tolerance (Kavi Kishor et al., 1995). Aromatic amino acid biosynthesis genes were predominantly repressed.

Amino acid degradation-related gene expression was more strongly repressed in Sullu than in SS2613. In SS2613, proline, tryptophan, and methionine degradation enzyme genes were down-regulated and in Sullu, proline degradation remained unchanged; inversely, cysteine and lysine degradation enzyme genes were repressed. Genes of gamma-aminobutyrate metabolism were strongly repressed in both clones under prolonged drought.

Transcriptomic and metabolite modifications related to proline, its analogues, and trigonelline synthesis in both cultivars are summarized in Fig. 6. Significant increases in L-4-hydroxoproline (HP) and N-methyl-L-proline (MP) contents were observed in leaves of both clones in response to drought treatment compared with the control (Fig. 6). In Sullu, proline and trigonelline contents were also increased. In SS2613, the increases were observed on days 15 and 28 AD, whereas in Sullu the accumulation of these compounds started later (day 28 AD), but also lasted longer (at least until day 49 AD). The increase in proline and proline analogues was linked to the up-regulation of P5CS and the down-regulation of proline dehydrogenase on day 28 AD (see Supplementary Table S4 at JXB online; Fig. 2). This is

<table>
<thead>
<tr>
<th>Table 3. Differential regulation of genes grouped to ‘bins’ using the MapMan software under drought stress compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bin</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>Photosynthesis</td>
</tr>
<tr>
<td>Major CHO</td>
</tr>
<tr>
<td>Minor CHO</td>
</tr>
<tr>
<td>Glycolysis</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
</tr>
<tr>
<td>Oxidative pentose phosphate cycle</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>Mitochondrial electron transport</td>
</tr>
<tr>
<td>Cell wall</td>
</tr>
<tr>
<td>Lipid metabolism</td>
</tr>
<tr>
<td>N-metabolism</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
</tr>
<tr>
<td>S-assimilation</td>
</tr>
<tr>
<td>Metal handling</td>
</tr>
<tr>
<td>Secondary metabolism</td>
</tr>
<tr>
<td>Hormone metabolism</td>
</tr>
<tr>
<td>Co-factor and vitamin metabolism</td>
</tr>
<tr>
<td>Tetrapyrrole synthesis</td>
</tr>
<tr>
<td>Stress</td>
</tr>
<tr>
<td>Redox</td>
</tr>
<tr>
<td>Polyamine metabolism</td>
</tr>
<tr>
<td>Nucleotide metabolism</td>
</tr>
<tr>
<td>C2-metabolism</td>
</tr>
<tr>
<td>Miscellaneous</td>
</tr>
<tr>
<td>RNA</td>
</tr>
<tr>
<td>DNA</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Signalling</td>
</tr>
<tr>
<td>Cell</td>
</tr>
<tr>
<td>Development</td>
</tr>
<tr>
<td>Transport</td>
</tr>
</tbody>
</table>
Fig. 4. (A) Heatmap of significantly regulated genes in ‘Photosystem and photosynthesis’ bins. Fold changes are expressed in log2 (stress/control).

(B) Partial heatmap of significantly regulated genes in ‘Carbohydrates’ bins. Fold changes are expressed in log2 (stress/control).
Fig. 4. Continued
in accordance with previous results showing that some genes involved in proline metabolism are induced by osmotic stress, while proline dehydrogenase activity is reduced (Sundaresan and Sudhakaran, 1995; Hien et al., 2003; Chen et al., 2009). Accumulation of proline and the quaternary amino acid derivative trigonelline is often recorded in response to hyperosmotic stress (Wood, 1999; Cho et al., 2003) and in response to a combined treatment of heat and drought stress (De Ronde et al., 2004; Simon-Sarkadi et al., 2005). Nevertheless, their role remains controversial, notably for proline. Some authors have detected high proline levels in susceptible cultivars (Premachandra et al., 1995; Sundaresan and Sudhakaran, 1995), while some others have observed the opposite trend (Hien et al., 2003). However, it generally appears that induction of proline is related to the degree of leaf osmotic stress (which is also suggested by our data) and the osmoregulation of genes involved in proline synthesis (see discussion above), as well as the contribution of some other solutes such as sugars or polyols to osmoprotection. In our experiment, an increase of 3.78 times in free proline content of Sullu leaves at 49 d AD compared with the control could suggest a contribution of this compound to osmotic adjustment. Lazcano-Ferrat and Lovatt (1999) came to the conclusion that proline is only an indicator of plant water status but not of tolerance or sensitivity of vegetative growth to water deficit, because they found an inverse relationship between proline levels and drought tolerance in Phaseolus species. A limited accumulation of proline in transgenic soybean showed that these plants are more susceptible to water stress (De Ronde et al., 2004; Simon-Sarkadi et al., 2005). Nevertheless, other roles have been suggested for proline, such as a singlet oxygen quencher and scavenger of OH- radicals in order to stabilize proteins, DNA, and membranes, or as a compatible solute (see Kavi Kishor et al., 2005, for a review).

Trigonelline has also been suggested to act as a compatible solute (Naidu et al., 1992; Oufir et al., 2009). This is in accordance with our data, since trigonelline content increased 2.39 times in Sullu leaves 49 d AD compared with the control. Wood (1999) suggested that its biosynthesis by the methylation of nicotinic acid may deplete the S-adenosyl-methionine pool and thus could limit oxidative stress-induced DNA methylation. It is interesting to note that other proline analogues also increased in Sullu, previously shown to be drought-tolerant, and although their quantity was minor compared with proline and trigonelline, a role in drought tolerance may be considered. To date, data about the implication of molecules such as HP and MP in abiotic stresses are scarce. N-methyl-L-proline has been reported to increase in several species in response to salinity or water stress (Naidu et al., 2000; Jones et al., 2006). The occurrence of MP analogues seems to be related to the treatment strength and the ability of the plant ecotype to adapt to various stressful habitats. Solomon et al. (1994)
have suggested that MP could protect the carboxylating activity of Rubisco against NaCl by stabilizing the hydration state. Hydroxyproline is an important component of the cell wall protein extensin. Several studies show an increase in this compound in the cell wall fraction in response to various abiotic stresses (Weiser et al., 1990;
Ueda et al., 2007), suggesting a role in increased synthesis or reconstitution of cell wall components. Among the various extensin genes, some of them were shown to be induced by a variety of abiotic stresses (Weiser et al., 1990; Yoshiba et al., 2001; Merkouropoulos and Shirsat, 2003). In our experiment, repression of the transcription of some extension genes occurred in response to drought stress in both cultivars, while transcription of one extensin gene was induced. Nevertheless, expression of these genes seems very specific to organs and up-regulation of some extensin genes involved in response to abiotic stresses are confined to roots (Merkouropoulos and Shirsat, 2003; Ueda et al., 2007). A transport of free HP from leaves to other plant organs could be suggested since it is possible for free proline. Actually, Ueda et al. (2007) reported an increase in the expression of a proline transporter in the apical region of barley roots under salt stress and suggested that free proline synthesized in other plant parts could be used for cell wall synthesis in the apical region of barley roots. Finally, glycine betaine levels were not affected by the drought treatment in the present experiment (results not shown).

Polyamine biosynthesis genes such as arginine decarboxylase and S-adenosylmethionine decarboxylase were induced at all time points in both clones. Polyamine concentrations (Fig. 7) decreased with time in control conditions in both cultivars. The level of the polyamine spermine (Spm) was higher in response to drought treatment in both clones as compared to the control plants, but the difference occurred sooner in SS2613. Such an increase in PAs level has been reported in response to abiotic stresses (reviewed by Groppa and Benavides, 2008), for example, drought stress (Capell et al., 2004; Liu et al., 2004; Bae et al., 2008), but the involvement of each kind of polyamine and their role still remain controversial. The polycationic nature of these molecules at physiological pH confers on them the property of binding negative charges in cellular

Fig. 7. Simplified scheme of polyamine synthesis in drought-exposed and control leaves of SS2613 and Sullu by transcriptomic (in blue, up-regulated genes) and metabolite-based approaches (analysed metabolites are underlined; blue, increased concentration; grey, unchanged concentration).
components; therefore, PAs have been suggested to increase membrane stability by decreasing their fluidity (Nayyar and Chander, 2004; Legocka and Kluk, 2005; Nayyar et al., 2005) and to act as free radical scavengers. Indeed, exogenous application of PAs reduced the hydrogen peroxide level in response to water stress (Tiburcio et al., 1994; Kubiš, 2003; Nayyar and Chander, 2004). Polyamines with a longer amine chain such as the tetramine Spm have been suggested to be more active in response to abiotic stress, which is in accordance with our results. Nevertheless, data about the relation between free polyamines and bound or conjugated polyamines are scarce and our results did not provide any information on bound or conjugated PAs. The hypothesis of an involvement in osmotic adjustment can be ruled out in our case since an increase of 49.8 and 51.5 nmol g⁻¹ DW in SSS2613 and Sullu, respectively, after 49 d of drought treatment represents only a slight contribution to osmotic balance. However, a possible role of free PAs may lie in signalling pathways. It has been speculated that polyamines may act as messengers in stomatal aperture modulation. Actually, PAs could regulate certain ion channels, especially Ca²⁺-permeable channels, and thus activate the K⁺ inward rectifier at the plasma membrane in guard cells by raising cytoplasmic Ca²⁺ concentration, which could stimulate stomatal closure (Liu et al., 2000; Oliver et al., 2000). This mechanism is specific to PAs and does not apply to any other compounds involved in their metabolism. Moreover, Spm having a higher net charge has been shown to be more efficient than putrescine and spermidine in this regulation, which is consistent with our results.

Osmotic stress does not seem to be the direct mediator of PAs accumulation, but the direct targets and receptors of osmotic signals, which induce polyamine accumulation, are not known yet (Li and Chen, 2000; Lefèvre et al., 2001). As described in the microarrays section (validated by real-time RT-PCR for ADC, Fig. 2), ADC as well as SAMDC genes involved in polyamine synthesis were up-regulated in both clones upon drought, and genes of γ-aminobutyrate metabolism involved in polyamine catabolism were strongly repressed in both clones. A predominant role of ADC in the stress response has already been shown (Soyka and Heyer, 1999; Legocka and Kluk, 2005; Liu et al., 2006).

The amino acid ornithine increased with time in control Sullu plants, but also in response to drought. This amino acid is notably a precursor in the synthesis of proline and its analogues, and PAs. Despite the fact that there are conflicting data about the quantitative implication of the synthesis of glutamate semialdehyde (GSA) from ornithine by ornithine-δ-aminotransferase (OAT) in response to abiotic stress (Delauney et al., 1993), this pathway, together with the glutamate pathway, was shown to play a great part in proline accumulation in response to osmotic stress (Sundaresan and Sudhakaran, 1995; Roosens et al., 1998; Lutts et al., 1999). Conflicting data about OAT activity could be related to PAs synthesis which can occur in response to osmotic stress.

Our results point to a greater implication of free proline and its analogues than PAs in the drought-stress response, as only Spm content increased 49 d AD. In the cultivar Sullu, proline content significantly increased upon drought stress, whereas changes in SS2613 were not significant.

In summary, under drought stress, concentrations of proline, proline analogues, and polyamines as well as inositol, galactinol, and galactose levels were higher in Sullu than SS2613, suggesting that these compounds are drought-responsive and putatively related to tolerance. A correlation between gene expression and metabolite data is not always clear, nor expected, as translational and post-translational regulation of enzyme activities can impact on metabolite synthesis.

Gene expression analysis revealed a larger distortion of photosynthesis and carbohydrate-related gene expression in Sullu than in SS2613. In comparison to previous results, which showed that in Sullu most photosynthesis and photorespiration related genes remained unchanged under drought stress (Schafleitner et al., 2007), down-regulation of these genes in our experiment might be related to the higher day temperatures. Down-regulation of these genes coupled with SPS and SS down-regulation may have consequences on metabolite production at the whole plant level, although no significant decrease in carbohydrates occurred at the leaf level studied. The allocation of sugars for tuber development may be reduced and the conversion of sucrose to starch in growing potato tubers is decreased by water deprivation and high temperature (Geigenberger et al., 2004). In the tubers, a significant decrease in total carbohydrates was observed in drought-exposed Sullu (Andre et al., 2009), reflecting different metabolite synthesis (and gene expression) patterns at the level of different organs. In the present experiment, the reduction of tuber yield is similar for Sullu and SS2613. Sullu was previously shown to be able to retain a high harvest index and allocate more resources to tubers under drought stress (Schafleitner et al., 2007), which led to this species being considered as a moderately agronomic drought-tolerant one, in comparison to other moderately tolerant species such as SA2563 which presented a different strategy against drought stress. This ability of Sullu to favour tuber formation under adverse conditions may be the reason why, in our field conditions, tuber yield of Sullu and SS2613 were similarly impaired, although the global regulation of gene expression looked more drastic in Sullu than in SS2613. Despite a clear tuber yield decrease when compared to previous experiments, Sullu was able to maintain an absolute yield three times higher than SS2613’s one.

**Conclusion**

Under drought stress, photosynthesis-related genes were strongly repressed in Sullu, whereas repression was less important and occurred later in SS2613. Similarly, a strong perturbation of carbohydrate-related genes was observed in Sullu. At the metabolite level, differential accumulation of osmotically active solutes was observed between the two cultivars; indeed, in Sullu, contents of galactose, inositol, galactinol, proline, and proline analogues were higher upon drought stress as compared to SS2613. These results point to different drought responses in the cultivars at the leaf level, with, however, similar tuber yield reductions.
The previously shown tolerant clone Sullu lost part of its tolerance under the present experimental conditions, as both control and drought-stressed plants suffered from elevated temperatures during the day. Experiments under growth chamber conditions which, in contrast to field trials, would allow different temperature regimes to be applied in drought trials, would result in a better dissection of the reactions to combined drought and elevated temperature stress.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Table S1. Microarray hybridization protocols.

Supplementary Table S2. Primer sequences used for real-time RT-PCR.

Supplementary Fig. S3. Lists of genes in the clusters of the hierarchical cluster analysis.

Supplementary Table S4. Lists of genes in the heatmaps.

Acknowledgements

The authors would like to thank Laurent Solinhac for his excellent technical assistance and Dr Torsten Bohn for his support in statistical analyses. This work was financially supported by the Ministry of Finance (Luxembourg).

References


