Time-course metabolic profiling in *Arabidopsis thaliana* cell cultures after salt stress treatment*

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Abstract

Salt stress is one of the most important factors limiting plant cultivation. Many investigations of plant response to high salinity have been performed using conventional transcriptomics and/or proteomics approaches. However, transcriptomics and proteomics techniques are not all-encompassing methods that can achieve exclusive insights into the metabolite networks contributing to biochemical reactions. Hence, the functions of the complex stress response pathways are yet to be determined, especially at the metabolic level. A time-course metabolic profiling with *Arabidopsis thaliana* cell cultures after the imposition of salt stress is reported in this study. Analyses of primary metabolites, especially small polar metabolites such as amino acids, sugars, sugar alcohols, organic acids, and amines, was performed by GC/MS and LC/MS at 0.5, 1, 2, 4, 12, 24, 48, and 72 h after a salt-stress treatment with 100 mM NaCl being the final concentration. The mass chromatographic data were converted into matrix data sets, which were subjected to data mining processes, including principal component analysis (PCA) and batch-learning self-organizing mapping analysis (BL-SOM). The mining results suggest that the methylation cycle for the supply of methyl groups, the phenylpropanoid pathway for lignin production, and glycine betaine biosynthesis are synergetically induced as a short-term response against salt-stress treatment. The results also suggest the co-induction of glycolysis and sucrose metabolism as well as co-reduction of the methylation cycle as long-term responses to salt stress.

Key words: *Arabidopsis thaliana*, cell culture, metabolome analysis, metabolomics, profiling, salt stress.

Introduction

Salt stress is one of the major environmental factors limiting worldwide crop productivities. High concentrations of NaCl may cause both hyperionic and hyperosmotic stress effects, which lead to a decline of turgor, disordered metabolism, and the inhibition of uptake of essential ions, as well as other problems in plant cells. Plants have many genetic sets for maintaining ion homeostasis. For metabolism, the synthesis of compatible solutes to activate adaptive responses is one such set. Recently, many salt-stress responsive genes and protein in plants have been identified by comprehensive transcriptomics and proteomics techniques (Kawasaki et al., 2001; Seki et al., 2002; Takahashi et al., 2004; Ueda et al., 2004; Yan et al., 2005; Wang et al., 2003; Parker et al., 2006). However, it is generally known that increases in mRNA levels do not always correlate to the increases in protein expression (Gygi et al., 1999), and proteins might not be enzymatically active even if they are translated (Sumner et al., 2003). In other words, the transcriptomics and proteomics techniques are not all-encompassing methods for achieving exclusive insights into metabolite networks contributing to biochemical reactions. Hence, the functions of their complex stress response pathways are yet to be determined, especially at the metabolic level.

Metabolomics is a comprehensive analysis during which a thorough metabolite profiling is performed. Metabolomics has emerged as a functional genomics methodology...
that contributes to our understanding of the complex molecular interactions in biological systems (Fiehn, 2002). In particular, metabolomics using time-course designs over short-term intervals can provide successive snapshots of metabolism status. Such methodology is useful in investigating the complex interacting mechanisms of cellular metabolic pathways in response to perturbations, such as salt stress. Only a few examples of salt-stress metabolomics studies have been performed. Recently, metabolic fingerprinting of the salt-stress response has been performed on NaCl-treated tomatoes by means of FT-IR spectroscopy (Johnson et al., 2003), which identified functional groups of potential importance in relation to the salinity response. Key spectral regions were identified within the FT-IR spectra corresponding to nitrile-containing compounds and amino radicals, however, the successive metabolic functions of the salt-stress response pathways were not elucidated.

Incidentally, the technical requirements of metabolomics for the present study should be mentioned. In metabolomics analysis, a precise analysis of the metabolites of concern is the most important factor. Efficient sample preparation and selective extractions, along with a combination of different analytical techniques are required. Because of the instability of a large variety of intracellular metabolites in practice, recovery of each metabolite of interest during the sample extraction procedure must be verified experimentally. This problem in sample preparation can be approached by stable isotope dilution techniques, in which stable isotopomers of the target compounds are used as an internal standard for the mass spectrometry analysis. An accurate comparative quantification of various nitrogen-containing metabolites, such as folate, S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH), and amino acids, has already been established in a previous study by means of LC/MS using isotopomers of the target metabolites as internal standards (Kim et al., 2005). An accurate method for the analysis of formate by SPME (solid-phase microextraction)-GC/MS, in which [13C]-formate was used as an internal standard (Kim et al., 2003) has also been developed. In the present study, a time-course metabolome analysis was conducted, in which major metabolites concerning one-carbon metabolism were focused on, to elucidate the metabolic modulation in a response of plant cells to salt stress. A time-course sampling up to the 72 h following the imposition of NaCl treatment was performed using Arabidopsis thaliana cell culture T87 as an experimental model cell. The reason for focusing on the metabolites related to one-carbon metabolism is explained below.

SAM and SAH are intermediates in the activated methyl cycle. In this cycle, SAM contributes to provide, under high salinity conditions, a methyl group to many metabolites, including important compounds such as glycinebetaine, methylated polyols, and polyamines. The genes encoding S-adenosyl-L-methionine synthase (SAMS) have been cloned from several plant species, and small gene families have been discovered in all cases (Peleman et al., 1989; Schröder et al., 1997). The genome of Arabidopsis thaliana contains four genes encoding putative SAMS. Lycopersicon esculentum and Catharanthus roseus possess at least three SAMS genes, that are differentially transcribed in response to various stress conditions, including salinity (Espartero et al., 1994; Schröder et al., 1997). Meanwhile, it has been reported that the mRNA of SAMS is down-regulated in high-salinity stress in Arabidopsis (Seki et al., 2002). Further, the protein levels of SAMS are down-regulated by salt stress (Yan et al., 2005). These results might conflict with the general understanding in which SAMS genes are up-regulated by salt-stress (Schröder et al., 1997). The reasons for the multiple conflicting results are unclear, thus, the way SAMS activates modulation against salt stress remains unknown. However, despite their importance and the well-conserved stress-related responses of SAMS genes in plant metabolism, very little is known regarding SAM endogenous levels under environmental stress conditions because SAM and SAH pools are difficult to quantify due to their labilities and low abundance (Hanson and Roje, 2001). Other metabolites, such as 5,6,7,8-tetrahydrofolate (THF), formate, serine, and glycine also have important roles in one-carbon (C1) metabolism (C1 transfer reactions) (Hanson and Roje, 2001). However, the role that C1 metabolism might play during plant adaptation to environmental factors is unknown. The mode of C1 metabolism against salt stress is an open question (Scott et al., 2000).

The special focus was on the profiling of polar small molecules, as well as metabolites related to one-carbon metabolism. The most important primary metabolites are polar small molecules. Target metabolites contain sugar, sugar alcohols, organic acid, amino acids, and amine. In addition, multivariate analysis of the data set obtained was performed to evaluate the results of these analyses and to interpret their physiological implications. Two common exploratory multivariate methods, principal component analysis (PCA) and batch-learning self-organizing maps (BL-SOM), were used.

Materials and methods

Plant material and culture conditions

Arabidopsis T87 cells were obtained from the RIKEN Bio Resource Center (Tsukuba, Japan). The T87 cells were grown in modified liquid LS medium (30 ml) in a 100 ml flask under continuous light exposure at a photosynthetic photon flux density (PPFD) of 55 μmol m⁻² s⁻¹ at 23 °C. In order to obtain ¹⁵N-labelled cells as an internal standard, T87 cells were grown in ¹⁵N-labelled medium containing R¹⁵NO₃ and ¹⁵NH₄¹⁵NO₃ under the same conditions. Every 7 d, 3 ml of mother cell suspension was transferred into 30 ml of fresh medium.
Salt-stress treatment
The cells were grown for 3 d first and then treated with 100 mM NaCl. The cells were sampled for analysis after 0.5, 1, 2, and 4 h and 0.5, 1, 2, and 3 d. Control cells were also sampled at the same point of time. The cells were harvested by filtration, immediately ground in liquid nitrogen and stored at −80 °C prior to analysis. Each data point is the mean of three replicates.

FW (fresh weight), DW (dry weight), RGR (relative growth rate) of T87 cells
Dried material of the cell cultures was obtained by heating the culture at 80 °C for 24 h. The initial FW and DW were defined to be 100% and RGRs were calculated accordingly (Zhao et al., 2004).

Metabolites extraction and quantitative analyses
Extraction, separation, identification, and measurement of SAM, SAH, folate, and amino acids by LC/MS analysis using isotopomers of the target metabolites as internal standards were performed according to the procedures previously reported by our group (Kim et al., 2005). In order to obtain the isotopomers, uniformly stable isotope-labelled T87 cells were cultured by using with 15N-labelled medium containing K15NO3 and 15NH4ClO4 for over 21 d. The same amounts of labelled cell pellets were added to each control cell and NaCl-treated cells as internal standards prior to the metabolite extraction step. Folate was extracted with 8 ml of extraction buffer (25 mM ammonium acetate, 2% sodium ascorbate, 20 mM 2-mercaptoethanol, pH 7.3). The extract was purified by affinity chromatography (Konings, 1999), then incubated with 1 ml of carboxypeptidase (1 unit) for 4 h at 30 °C. After freeze-drying, the sample was dissolved in 0.2 ml of 15 mM ammonium acetate buffer (pH 7.3) and stored under argon at −80 °C until analysis. For SAM and SAH extraction, the aliquot was suspended in 3 ml of H2O and lysed by vortexing for 30 s. Protein from clarified lysates was then precipitated with ethanol (final concentration of 70%) at −20 °C for 15 min, and removed by centrifugation (13,000 g, 20 min, 4 °C). The extract was purified by solid phase extraction using a C-18 Sep-Pak cartridge (Waters, Milford, MA, USA). Amino acids were extracted with 1 ml of methanol:H2O:chloroform (2.5:1:1 by vol.) solution. Subsequently, the methanol/water phase was dried in a centrifuge concentrator overnight, and precolumn derivatization of the sample was carried out using 5-dimethylaminonaphthalene-1-sulphonyl chloride. Stable isotope dilution analysis of folate, SAM, SAH, and amino acid was performed by LC/MS according to the procedures previously reported by our group (Kanaya et al., 2005). The ratio of SAM to SAH was represented as a ‘methylation index’ (SAM/SAH), which is thought to be an important dimensionless parameter for the evaluation of endogenous methylation activity (Fojtova et al., 1998; Stabler and Allen, 2004). For formate extraction, NaCl-treated cells and the control cells were harvested by filtration, and were immediately ground in liquid nitrogen and stored at −80 °C until assayed. The aliquots (40 mg) were overlaid with 1 ml of 200 mM KOH solution containing 40 μmol [13C]-formate. The sample was mixed by vortexing for 30 s, centrifuged at 16,000 g for 10 min at 4 °C, and the supernatant was used for formate analysis. After derivatization with methyl formate under acidic conditions, head-space SPME and GC/MS procedures were performed, according to the method previously reported (Kim et al., 2003).

For other metabolite analyses, 100 μl of aliquot was extracted with 1 ml of a mixed solvent of methanol/water/chloroform (2.5:1:1 by vol.). Sixty microlitres of ribitol solution (0.2 mg ml−1) was added as an internal standard. Extraction was performed at 37 °C with a mixing frequency of 1200 rpm, using a thermomixer compact (Eppendorf AG, Germany). The solutions were then centrifuged at 16,000 g for 3 min. Polar phase (0.9 ml) was transferred into a new Eppendorf tube, and 0.4 ml of water was added. The well-mixed content of the tube was centrifuged at 16,000 g for 3 min. The methanol/water phase was dried in a centrifuge concentrator for 4 h, followed by lyophilizing for 4 h. Derivatization was carried out by adding 50 μl of methoxyamine hydrochloride (20 mg ml−1) in pyridine and shaking at 30 °C for 90 min. The sample was trimethylsilylated for 30 min at 37 °C by adding 100 μl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). One microlitre of the derivatized sample was injected at a split ratio of 1:25 into a GC/MS system consisting of an AS 3000 autosampler, a TRACE GC gas chromatograph equipped with a 30 m×0.25 mm i.d. fused silica capillary column coated with CP-Sil 8 CB low bleed (varian), and a TRACE DSQ mass spectrometer (Thermo Electron Co., Waltham, MA, USA); the injection temperature was 230 °C. The oven temperature was sustained at 70 °C for 5 min, then raised by 6 °C min−1 to 310 °C, and held for 4 min. Both the interface and the ion source temperature were set at 250 °C. Helium was used as the carrier gas at a flow of 1 ml min−1. Mass spectra were recorded at 2 scans s−1 at a scanning range of 50–600 m/z. Peak finding and quantification of selective ion traces were accomplished using Xcalibur software and Automated Mass Spectral Deconvolution and Identification System (AMDIS) software.

Peak identification was confirmed by comparison of the reference compounds and the use of the NIST98 library. In addition, identifications of several metabolites were performed by the direct comparison of the sample mass chromatogram to those of commercially available standard compounds, which were obtained by a similar TMS derivatization and GC-MS analysis. In addition to the 14 amino acids analysed by LC-MS, 29 amino acids, sugars, sugar alcohols, amines, acids, and hydroxy-acids were found (see supplementary Table S2 at JXB online).

Principal component analysis (PCA)
In Arabidopsis thaliana T87 cells, 47 metabolites were identified by GC/MS and LC/MS (see supplementary Table S1 at JXB online), and the profiled metabolite data were analysed using PCA. PCA is one of the most popular clustering methods for exploratory data analysis requiring no knowledge of data structures (Goodacre et al., 2000). The PCA was performed in accordance with the customary manner using Pirouette software (Infometrix, Woodinville, WA) with mean-centre preprocessing. Data were visualized by using the principal component score and loading plots. Each point on the scores plot represents an individual sample, and each point on the loading plot represents a contribution of individual metabolite to the score plot. Biochemical components responsible for the differences between samples detected in the scores plot can accordingly be extracted from the corresponding loadings.

Batch-learning self-organizing map analysis (BL-SOM)
For BL-SOM analysis, relative values of the normalized metabolite levels were used. The highest level in the time-course for each metabolite was set to 1, which gave relative levels for each time-course sample. BL-SOM analysis was conducted according to a previously reported method (Kanaya et al., 2001). BL-SOM is an improved method over a simple SOM, in which the initial weight vectors are set by PCA, and the learning process is designed to be independent of the order of input of vectors. Hence the results are reproducible.

Results
Effect of salt on Arabidopsis thaliana T87 cell growth
Arabidopsis thaliana T87 cells, which were established from the ecotype Columbia, were used. In a recent study,
T87 cells treated with 100 mM NaCl were used as a model plant system for transcriptional regulation investigation in response to high salinity (Takahashi et al., 2004). Therefore, 100 mM NaCl was also used for salt stress. Under the control condition, culture growth of T87 cells displayed a lag phase of 60 h, a log phase of rapid growth of about 84 h, then a stationary phase in the normal conditions (data not shown). Thus, in order to investigate the effect of NaCl on cell growth during the log phase, cells cultured for 3 d were treated with a final concentration of 100 mM NaCl, and the FWs and DWs of the cells were measured to give RGR curves over 3 d (Fig. 1). Although RGRs of NaCl-treated cell cultures were lower than those of the control cell cultures, their FWs and DWs still increased under salt stress in the T87 cell line cultures.

Time-course change of metabolite profile following the imposition of salt stress

The metabolites listed in supplementary Table S1 (at JXB online) were analysed at eight different time points up to a maximum time of 72 h after the imposition of salt stress. The data obtained were subjected to PCA to outline the difference between each time-point sampling at the metabolome level. The results are indicated by principal component score plotting (Fig. 2). The abscissa represents the principal component 1 (PC1) score, while the ordinate represents the principal component 2 (PC2) score. Each plot in Fig. 2 implies the corresponding metabolome. PCA revealed that the two highest ranking principal components accounted for 89% of the total variance within the dataset. Principal component 3 and all subsequent principal components did not provide any further differentiation between the sample types. The first principal component, accounting for 74.3% of total variance, resolved the time series of both early and late salt-stress responses. In particular, the metabolome 72 h after the salt treatment is clearly standing out from the other metabolomes in PC1. The second principal component, accounting for 14.2% of total variance, resolved the time series of only early salt stress responses. The metabolome 24 h after the salt treatment is separated from the others in PC2. In order to investigate the contributors of the principal components of the PCA further, the metabolic loadings in principal component 1 (PC1) and principal component 2 (PC2) were compared. The significant metabolites for principal component 1 were lactate and sucrose. The results suggest that lactate and sucrose are abundantly accumulated in the cells 72 h after salt treatment. In principal component 2, the corresponding loading was positive for tyrosine, sucrose, tryptophan, and phenylalanine, while negative for glycerol and inositol. The results suggest that the accumulation levels of tyrosine, sucrose, tryptophan, and phenylalanine in the cells 24 h after salt treatment are lower compared with other cells. The results also suggest that glycerol and inositol are abundantly accumulated 24 h after treatment.

Time-course response of endogenous levels of the metabolites related to one carbon metabolism following NaCl treatment

The significant metabolites concerning one carbon metabolism, including folate, SAM, SAH, and formate, were analysed based on a stable isotope dilution comparative quantification, using isotopically labelled metabolites or compounds as the internal standards. That quantification system provided notably high fidelity and reproducibility to observe small changes in metabolite level. In the short-term period (0–4 h), SAM was increased at all times following NaCl treatment, while the levels of SAH were not affected by salt (Fig. 3). In particular, 1 h after treatment, the level of SAM was drastically increased. Therefore, a very early increase in the methylation index (the ratio of SAM/SAH) occurred as a consequence of salt treatment.

In the long-term following NaCl treatment (12–72 h), the SAM endogenous level gradually decreased to less than 1.0 times the relative peak ratio according to the
elapsed time (Fig. 3). By contrast, the endogenous level of SAH gradually increased after 12 h following the NaCl treatment and finally reached over 1.5 times the relative peak ratio (Fig. 3). Hence, the methylation index constantly decreased after 12 h following NaCl treatment. Control cells showed a continuous increase in formate levels throughout the time-course of culturing (Fig. 4). However, at the time points of 12 h and 24 h, the comparative ratios of endogenous levels of formate in the NaCl-treated cells compared with the control cells were similar to those observed in the control. The comparative ratio of formate endogenous levels in NaCl-treated cells rose slightly 48 h after the imposition of salt stress (Fig. 4). Among the folates observed in Arabidopsis T87 cells, 5-methyltetrahydrofolate was predominant. The endogenous level of 5-methyltetrahydrofolate was not affected throughout the period after the imposition of NaCl treatment (data not shown).
Classification of all metabolites according to the time-course fold change pattern of their accumulation

BL-SOM analysis was conducted to classify the metabolites according to their patterns of accumulation (Fig. 5). All metabolites were classified into four groups according to the similarity of their fold change pattern in endogenous accumulation levels. Group A and Group B contain metabolites that increased in the short-term period following NaCl treatment while decreasing in the long-term. Metabolites that increased in the long-term period were classified into Group C. The other metabolites that indicated no remarkable changes were categorized into Group D. Group A includes the methylation index (SAM/SAH), ethanolamine, and cysteine that is the precursor of methionine biosynthesis. Group B contains aromatic amino acids such as tyrosine, tryptophan, and phenylalanine. Both groups show only slightly differences based on the initial time-points, in which their endogenous levels start to rise (30 min or 1 h). Group C contains metabolites related to glycolysis and sucrose metabolism. Metabolites in Group D have little obvious functional similarity with each other.

Discussion

The time-course metabolic profiling of salt-stress response in Arabidopsis thaliana T87 cell culture, in which the metabolites’ dynamic levels are listed in detail, is presented for the first time. As an essential step of data mining, a principle component analysis (PCA) was conducted to determine the fundamental data structure, and to verify the reliability of the present experiment. PCA in the present study allows easy visualization of complex data, and the time series effects mainly contribute to the total variance within the data set (Fig. 2). The PCA results clearly show that the variances among the same time point samples were not so great. The negligible inter-experimental variation demonstrates the robustness of the present experimental system, especially with respect to reproducibility.

In order to mine the data set obtained from the time-course design, a clustering based on similarities of the fold change pattern was selected, because functionally related metabolites might be classified into the same cluster. A batch-learning self-organizing mapping (BL-SOM) algorithm was conducted to classify the metabolites into four clusters indicating similar profiles of metabolite fold change (Fig. 5). Some interesting correlative metabolic networks for understanding the complex reaction mechanism against salt stress were found through the mined data. These results are summarized in Fig. 6. The BL-SOM results show that the fold change patterns of Group A and of Group B are so similar that further discussion about them is combined. The results of Group A and Group B suggest that salt stress leads to a co-induction of

Fig. 5. Self-organizing map (SOM) analysis of metabolite data of differential response with respect to the time series. Data were analysed as described in the Materials and methods. Neurons were arranged in two-dimensional lattice. Metabolites with similar level profiles are clustered in the same neuron of a 4×3 matrix SOM. Neurons contain from 1 and 8 metabolites. Four neurons selected with different level patterns for SOM analysis were clustered. A, B, C, and D were designated as Group 1, 2, 3 and 4, respectively, in the Results and Discussion. Comparative changes in metabolite levels are shown in the boxes (1, 0.5 h; 2, 1 h; 3, 2 h; 4, 4 h; 5, 12 h; 6, 24 h; 7, 48 h; 8, 72 h) as red (most increased), orange (increased) or blue (decreased). No change in metabolite level is shown in the box as white. * Methylation is the ratio of SAM to SAH.
aromatic amino acids and methylation index. Subsequently aromatic amino acids and methylation index were co-reduced after 12 h from the imposition of salt stress. SAM levels increased and subsequently decreased to one-third of the initial level (Fig. 3). This is consistent with the previous studies. Despite the importance of SAM in salt tolerance, SAMS mRNA levels are eventually downregulated by salt stress in Arabidopsis (Seki et al., 2002).

The level of cysteine, which is an important precursor for methionine synthesis, showed a similar time-course fold change pattern to the methylation index. The combined induction of aromatic acids and methylation activity implies that lignin biosynthesis should be induced by salt stress. Increased lignin levels should contribute to the enhancement of cell wall strength. Among the aromatic amino acids, tryptophan has been shown to be a positive feedback activator of phenylalanine and tyrosine biosynthesis (Bentley, 1990). Tryptophan biosynthetic enzymes are known to be up-regulated in response to biotic and abiotic stress. For example, anthranilate synthase α, tryptophan synthase α, and tryptophan synthase β are inducible by acifluorfen (oxidative stress-inducing herbicide) treatment in Arabidopsis (Zhao et al., 1998). According to the present results, the co-induction of aromatic amino acids is consistent with the previous study results.

In addition, Group A contained ethanolamine (Fig. 5A). Ethanolamine is essential for the synthesis of phosphoethanolamine and phosphatidylethanolamine as well as...
choline and glycinebetaine, in some species. Glycinebetaine is important because it has a strong osmoprotectant properties and confers tolerance to salinity, drought, and other stresses (Rhodes and Hanson, 1993; Sakamoto and Murata, 2000). In Arabidopsis, the silencing of the phosphoethanolamine N-methyltransferase gene results in salt hypersensitivity (Mou et al., 2002). Based on the correlative analysis of metabolite level patterns, it is suggested that the methylation pathway and glycinebetaine biosynthesis pathway are systematically co-ordinated as an initial response to salt stress.

Long-term exposure of Arabidopsis T87 cells to salinity (more than 12 h) led to reduction in metabolites, which were initially responsive (Fig. 5A, B). In addition, glycolysis and sucrose metabolism were co-induced at the 72 h time point as shown in Group C (Fig. 5C). The observed metabolites with an osmoregulate function, such as sucrose and fructose, are also involved in Group C (Fig. 5). Sugar accumulation would act as an adaptation solute contributing to balancing the osmotic strength both inside and outside the cells. In detail, this would prevent cellular dehydration, and provide a source of energy essential under saline conditions to fuel a more active cell metabolism. Therefore, it is speculated that co-induction of the metabolites involved in glycolysis and sucrose metabolism at the 72 h time point would be one such tolerant mechanism.

A previous study reports that the long-term effects resulting from accumulation of salt in plants involve reduced photosynthetic activities, elevated oxidative stress leading to anaerobic conditions, and metabolic damage (Abbasi and Komatsu, 2004). In addition, the metabolites involved in glycolysis might be closely related to the adaptive response to oxidative stress. The decrease of the methylation index would affect photosynthetic activity because SAM-dependent methylation is essential to chlorophyll biosynthesis. A previous report that indicated that SAMS genes were down-regulated by salt stress (Seki et al., 2002) is consistent with this idea.

Fumarate levels were increased in the long term, while the levels of tyrosine and phenylalanine decreased (Fig. 5C). Fumarate must be the break-down product of phenylalanine and tyrosine in amino acid catabolism, as naturally expected. Moreover, among the amino acids analysed, only alanine was increased at the 72 h time point for T87 cell cultures with the presence of salt (Table 1). Under salt stress, alanine is accumulated as a storage form of pyruvate.

The effect of NaCl on 5-methyltetrahydrofolate endogenous levels in Arabidopsis T87 cells has been investigated for the first time. Interestingly, under this plant model system, salt stress did not lead to the significant change in 5-methyltetrahydrofolate endogenous levels (data not shown). To date, the true source of formate in plant tissues under the stress has not been established. Recently, it has been reported that suppression of formate dehydrogenase in Solanum tuberosum increases the steady-state levels of formate (Ambard-Bretteville et al., 2003). However, this report did not indicate the possible source of formate supply. A possible source of formate supply under salt stress is suggested in this study. Formate may be produced during photosynthesis by the non-enzymatic decarboxylation of glyoxylate by hydrogen peroxide. Conditions leading to glyoxylate decarboxylation may arise if the transamination of glyoxylate to glycine fails and glyoxylate accumulates, i.e. when there is a deficiency of amino donors (Igamberdiev et al., 1999). In the long-term response to salt, malate and formate were increased, but most amino acids were decreased in these studies. Malate is an intermediate in the glyoxylate cycle, and is essential for maintaining osmotic pressure and charge balance, and thus is involved in the regulation of stomatal apertures (Emmerich et al., 2003).

As described above, the metabolic profiling of Arabidopsis thaliana T87 cells using a time-course design in response to salt stress enabled the different systematic mechanisms in the complex metabolic events induced by

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**Table 1. Time-course change of amino acid levels in 100 mM NaCl-treated T87 cells**

Data are normalized to the mean response calculated for the control cells of each measured batch. Values are mean ± SEs (n=3).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.77±0.05</td>
<td>0.74±0.09</td>
<td>0.80±0.06</td>
<td>1.14±0.17</td>
<td>0.70±0.02</td>
<td>0.53±0.06</td>
<td>0.59±0.08</td>
<td>0.38±0.05</td>
</tr>
<tr>
<td>Asn</td>
<td>1.01±0.11</td>
<td>0.97±0.02</td>
<td>1.17±0.11</td>
<td>0.95±0.06</td>
<td>0.74±0.08</td>
<td>1.00±0.02</td>
<td>0.87±0.03</td>
<td>0.46±0.07</td>
</tr>
<tr>
<td>Ser</td>
<td>0.79±0.02</td>
<td>0.82±0.04</td>
<td>0.94±0.12</td>
<td>0.90±0.01</td>
<td>0.82±0.01</td>
<td>0.82±0.02</td>
<td>0.88±0.04</td>
<td>0.80±0.00</td>
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<tr>
<td>Gly</td>
<td>0.91±0.01</td>
<td>0.85±0.02</td>
<td>0.96±0.01</td>
<td>0.82±0.03</td>
<td>0.78±0.01</td>
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<td>0.97±0.03</td>
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<td>Ala</td>
<td>0.92±0.04</td>
<td>0.86±0.09</td>
<td>0.85±0.03</td>
<td>0.91±0.04</td>
<td>0.71±0.00</td>
<td>1.31±0.06</td>
<td>1.30±0.03</td>
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<tr>
<td>Glu</td>
<td>0.46±0.02</td>
<td>0.55±0.02</td>
<td>0.48±0.02</td>
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<td>0.69±0.07</td>
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<td>0.61±0.05</td>
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<tr>
<td>Gin</td>
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<td>0.61±0.04</td>
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<td>0.37±0.04</td>
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<tr>
<td>Thr</td>
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<td>1.04±0.04</td>
<td>1.08±0.06</td>
<td>0.98±0.01</td>
<td>0.89±0.06</td>
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<tr>
<td>Pro</td>
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<td>0.80±0.01</td>
<td>0.95±0.03</td>
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<tr>
<td>Val</td>
<td>0.81±0.01</td>
<td>1.01±0.06</td>
<td>1.01±0.11</td>
<td>1.01±0.04</td>
<td>0.85±0.03</td>
<td>0.69±0.01</td>
<td>0.70±0.01</td>
<td>0.43±0.05</td>
</tr>
<tr>
<td>Trp</td>
<td>1.31±0.06</td>
<td>2.34±0.09</td>
<td>2.18±0.05</td>
<td>1.11±0.04</td>
<td>1.01±0.07</td>
<td>0.37±0.02</td>
<td>0.45±0.03</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>Ile</td>
<td>1.07±0.04</td>
<td>1.21±0.03</td>
<td>1.20±0.14</td>
<td>1.12±0.03</td>
<td>0.92±0.04</td>
<td>0.85±0.05</td>
<td>1.04±0.04</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Leu</td>
<td>1.11±0.01</td>
<td>1.15±0.03</td>
<td>1.22±0.17</td>
<td>1.20±0.00</td>
<td>0.89±0.04</td>
<td>0.56±0.03</td>
<td>0.72±0.02</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>Phe</td>
<td>1.47±0.06</td>
<td>1.88±0.08</td>
<td>1.60±0.01</td>
<td>1.31±0.05</td>
<td>0.66±0.03</td>
<td>0.29±0.00</td>
<td>0.50±0.01</td>
<td>0.61±0.10</td>
</tr>
</tbody>
</table>
salinity to be elucidated. The approach presented in this study should provide useful information by proposing possible working hypotheses about metabolic network modulation against imposition of perturbations. The potential of ‘time-course metabolic profiling’ as a powerful tool for analysing an important piece of the puzzle in functional genomics and systems biology has been demonstrated through this study.

Supplementary data

Supplementary data are available at JXB online. Table S1: Identified metabolites in LC-MS and GC-MS chromatograms from Arabidopsis thaliana T87 cell extracts. Table S2: Time-course change of metabolite levels in 100 mM NaCl-treated T87 cells.

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