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PULSED ELECTRIC FIELD-INDUCED CELL PERMEABILISATION OF POTATO TISSUE LEAD TO SUSTAINED METABOLIC CHANGES

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Abstract:

Metabolite profiling was used to characterize stress responses of potato tissue subjected to reversible electroporation, providing insights on how potato tissue responds to a physical stimulus such as pulsed electric fields (PEF), which is an artificial stress. Wounded potato tissue was subjected to field strengths ranging from 200 to 400 V/cm, with a single rectangular pulse of 1 ms. Electroporation was demonstrated by propidium iodide staining of the cells nucleae. Metabolic profiling of data obtained through GC/TOF-MS complemented with orthogonal projections to latent structures (OPLS) clustering analysis showed that 24 h after the application of PEF, potato metabolism shows PEF-specific responses characterized by the changes in the hexose pool that may involve starch and ascorbic acid degradation.

Key words: electroporation, stress response, cell membrane recovery

1 Introduction

In recent years, there has been an increasing interest in the use of pulsed electric field (PEF) technology due to its potential to induce non-thermal permeabilization of cell membranes. Depending on the cell properties (i.e. size, conductivity, shape and orientation) and electropulsation parameters (i.e. field strength, duration and number of pulses), the application of PEF may cause lethal damage to cells due to irreversible loss of cell membrane permeability properties, leakage of cytoplasmic contents and lysis (Aronsson et al. 2001).

By strict control of the electropulsation parameters, permeabilization may evade affecting the cell viability. In this non-lethal version of the PEF technique, physiological responses to PEF-induced stress are still largely unknown. When using PEF as stressor, interesting findings for the pharmaceutical field, such as the increased yields of a cytostatic compound in cell culture of *Taxus chinensis* (Ye et al. 2004), and for the food science and nutrition field, such as the increased concentration of antioxidants and phytoesterols from oil seeds and fruits (Guderjan et al. 2005, 2007; Balasa 2007), encourage deeper metabolic studies that can help to a better understanding of the complex, dynamic metabolic behavior of plant tissues subjected to this novel (i.e. not present in nature) stress condition.

Metabolic profiling, which refers to the non-biased, comprehensive analysis of soluble cellular metabolites from a biological system (Dunn et al. 2005) has been applied to the study

of plant metabolism over the past several years (Wishart 2008). Global metabolic profiling is a research tool that can detect and monitor unidentified compounds as well as identified metabolites that play important roles in metabolism and physiology (Kaplan et al. 2004) and, in the context of this work, responses to stress.

We have performed metabolic profiling analysis using gas chromatography-mass spectrometry/time-of-flight (GC/TOF-MS) aiming at identifying compounds that exhibit PEF-specific responses when the electric pulses are applied to cause electroporation to the wounded potato tissue. This study allows discriminating between the potatoes response to PEF treatment against that of wounding alone, giving insights on to what extent PEF overlaps with other stresses. We suggest here that PEFs may cause effects in the metabolome of potato, which might be linked with cellular events following electroporation and recovery of cell membrane functionality.

2 Materials and methods

2.1 Preparation of samples and electrical treatments

Medium-sized potato tubers (13.0±3.0 cm in length, 7.0±0.5 cm diameter) harvested in the south of Sweden were manually washed and peeled. Slices, 15 mm thick were cut from the centre of a single tuber. The slices were oriented perpendicular to the major tuber axis. Rectangular cross-section samples, 15 mm long and 6.0 mm wide, were obtained from the phloem parenchyma tissue of the slices using a pair of parallel sharp blades. Immediately after cutting, the sample was rinsed with distilled water (at 20ºC) and gently blotted with medical wipes to remove the excess of water from the sample surface.

Electric pulses were delivered to the rectangular samples through two parallel, flat stainless steel electrodes (35 mm long and 10 mm wide) separated 6 mm. Electric pulses were delivered axially to the tissue using a Cellect electromanipulation instrument (BioFusion SCI AB, Lund, Sweden). Samples were treated at varying voltages (120, 180, 240 and 300 V, which corresponds to the electric field strength in air of 200, 300, 400 and 500 V/cm) with 1 ms rectangular pulses.

2.2 Fluorescence microscopy

The effect of electrical treatments on cell membrane electroporation was tested by fluorescence microscopy. Rectangular samples of potato parenchyma were incubated for 20 h at 4ºC in a 25 µM solution of propidium iodide (Sigma, λ_{ex} = 536 nm; λ_{em} = 617 nm) in 10 mM PBS buffer, pH 7.5. After the incubation time, samples were PEF-treated. Untreated samples were used as negative controls. After treatment, a 1 mm thick cross section was cut from the rectangle and immediately placed in 5ml PBS buffer for 3 min under slight agitation to wash away the excess of dye and starch from its surface. Microscopic observations of the cross section were made with a Nikon inverted fluorescence microscope (Nikon Co., Kawasaki, Japan) at a magnification of 10X. Five slices from four different tubers were examined at each experimental condition.

2.3 Metabolic profile evaluation of the effect of electrical treatments

24 hours after PEF application, sample preparation, extraction, derivatization and GC/TOF-MS analysis of metabolites was performed as described by Gómez Galindo et al. (2009).

2.4 Processing of metabolites data

Non-processed MS data from GC/TOF-MS analysis were exported in NetCDF format to MATLAB 7.0 (Mathworks, Natick, MA, USA), as described by Gómez Galindo et al. (2009). The resolved MS spectra were matched against reference mass spectra using the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral

search program 2.0. Peaks were identified or annotated based on retention indices (RIs) and the reference mass spectra comparison to the following databases: commercial database from NIST v. 2.0, the Umeå Plant Science Centre (Umeå, Sweden) in house metabolomics database and the Max Plank Institute for Molecular Plant Physiology (Postdam, Germany) in house metabolomics database.

2.5 Statistical analysis for metabolic profiling

After peak annotation, the obtained data matrix after GC/TOF-MS analysis was normalized using the concentrations of the 11 internal standards. After normalization, the matrix was used for multivariate analysis. The orthogonal projections to latent structures (OPLS) method was performed as a supervised multivariate analysis with SIMCA-P+ 11.5 software (Umetrics AB, Umeå, Sweden).

In order to narrow down the variables (metabolites) most influential to the separation of the groups in the OPLS model, weight plots (w*c[2]O vs. w*c[1]P) showing relationships between all factors and responses were used.

3 Results

3.1 Electroporation

The electroporation experienced by the samples at the applied PEF conditions is demonstrated by the penetration of propidium iodide in the cells and the staining of their nucleus (Fig 1), which can be clearly seen in the pictures as bright circles inside the cells.

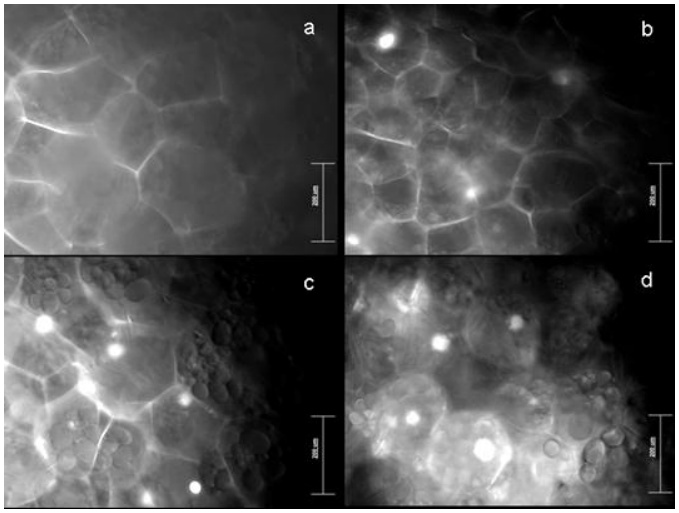

Fig. 1. *Typical experimental results from microscopic observations of potato tissue treated with electric fields and stained with propidium iodide, as indicated in the Materials and methods section. The red channel of the original images is shown. (a) untreated sample (negative control), (b) samples treated with a single 1 ms pulse of 200 V/cm, (c) samples treated with a single 1 ms pulse of 300 V/cm, (d) samples treated with three 1 ms pulse of 500 V/cm (positive control).*

3.2 Cluster analysis of metabolic profiles

After untargeted GC/TOF-MS analysis, the H-MCR process extracted 154 metabolite peaks and their mass spectra. Using the databases, 64 peaks were identified or annotated as known metabolites.

OPLS was applied to all metabolic peaks for all treatments (Fig 2). Data obtained at 400 V/cm were not included because cell death and microbial contamination were suspected. Twenty four h after the application of the treatments, 3 clusters were clearly observed, the metabolites from fresh tissue, those of the wounded tissue and those of the PEF-treated tissue, without any discrimination between the intensities of the applied electric field.

3.3 Specific responses to wounding and PEF-stress

The OPLS clustering was further analyzed as follows: (i) the fresh tissue (Time 0) differentiated from the tissue 24 h after wounding. (ii) The tissue 24 h after wounding from the tissue 24 h after it was wounded and PEF-treated. In this way, metabolites contributing to the described clustering, differentiating metabolic responses between wounding specific effects and PEF specific effects could be analyzed. As detailed in the Materials and methods

section, weight and variable importance plots together with ANOVA analysis were used to select the discriminative metabolites.

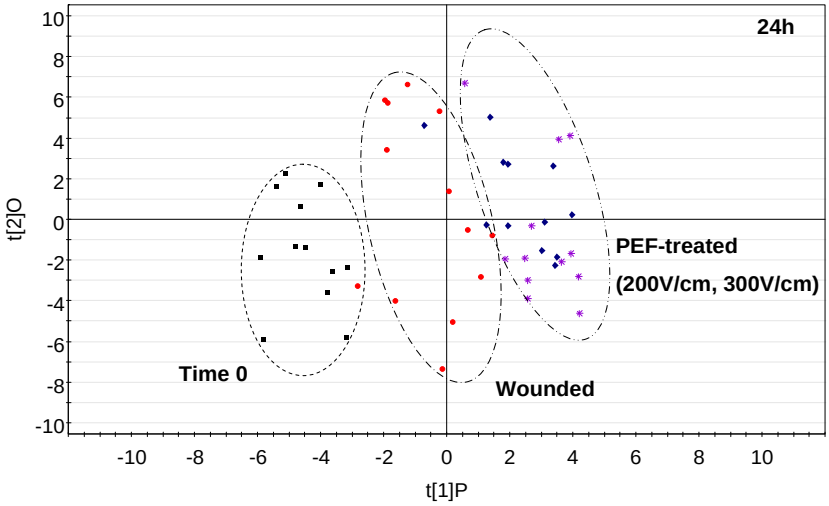

Fig. 2. *Orthogonal projections to latent structures (OPLS) analysis of metabolites from samples of potato parenchyma subjected to different treatments. Samples were either (■) untreated (Time 0), (●) wounded or (◆, ✦, ▲) wounded and PEF-treated at varying voltages, as described in the Materials and methods section. Clustering analysis of samples is reported 24 h after treatments.*

Metabolites showing to be significantly affected 24 h after wounding are highlighted with a circle in a simplified metabolic pathway map (Fig 3). Only known compounds are reported in Fig 3. Metabolites are annotated red for increased concentration and blue for decreased concentration.

Differences of metabolites obtained 24 h after the tissue was wounded and PEF treated are highlighted in Fig 3 as PEF specific effects. When the wounded tissue has been subjected to an electric field, its overall metabolic response is apparently the same as the wounding response regarding the changes in the amino acid pool and the tendency to increase the levels of sterol and galactosyl glycerol-like compounds. Interestingly, the metabolic response after PEF stress differentiates from wounding in the hexose pool. There is an accumulation of sucrose and fructose as well as an accumulation of threonic acid, a degradation product of ascorbic acid, which will eventually contribute to the hexose pool (Loewus 1999).

Another interesting deviation of the wounding specific response when PEF is applied is the high level of quinate and low level of chlorogenic acid. Chlorogenic acid constitutes up to 90 % of the total phenolic content of potato tubers and is an important defence-related compound, protecting potatoes from attack by phytopathogens and insects (Friedman 1997), and it is accumulated in wound-healing potatoes as a part of their defence mechanism. Surprisingly, the concentration of chlorogenic acid of the PEF-treated tissue is similar to that of the fresh potato (Time 0). Visual assessment of the potato samples did not reveal differences in the levels of enzymatic browning between the wounded and the PEF treated tissue that could justify the differences in the levels of chlorogenic acid.

4. Discussion

Our results demonstrate that 24 h after the application of PEF, potato metabolism shows PEF-specific responses characterized by the changes in the hexose pool that may involve starch and ascorbic acid degradation. In potatoes, accumulation of sucrose and other hexoses has

been reported to be a common stress response when the stressor, such as cold temperatures and drought, targets the plasma membrane and may produce electrolyte leakage (Herppich et al. 2001; Blenkinsop et al. 2004). This sugar accumulation has been directly correlated with starch degradation (Blenkinsop et al. 2004). Increase in soluble sugars may play a role in osmoregulation and possibly also in the activation of respiratory metabolism (Espen et al. 1999). Osmoregulation might be very relevant for the case of PEF stress as cells in the tissue might experience a significant osmotic imbalance after pulsing but not after wounding, with different metabolic consequences.

With the application of PEF, opening of pores in the plasma membrane will result in the efflux and influx of polar molecules. After the pulse application, resealing process takes place in a time scale of seconds or minutes. After resealing, the cell membrane recovers its properties in a long term physiological process that may take from several hours to days (Teissie et al. 2005). This process might involve ATPase activity, which uses the chemical energy of ATP, helping the cells to take up the leaked ions against the concentration gradient (Arora and Palta 1991). This hypothesis of the high metabolic energy requirements during the recovery process is supported by our results suggesting that the wounded tissue subjected to PEF is actively mobilizing its carbon energy sources that might involve starch and ascorbic acid degradation, contributing to the hexose pool. The time scale of hours where the reported PEF-specific effects took place agrees with the time scale needed for the recovery process.

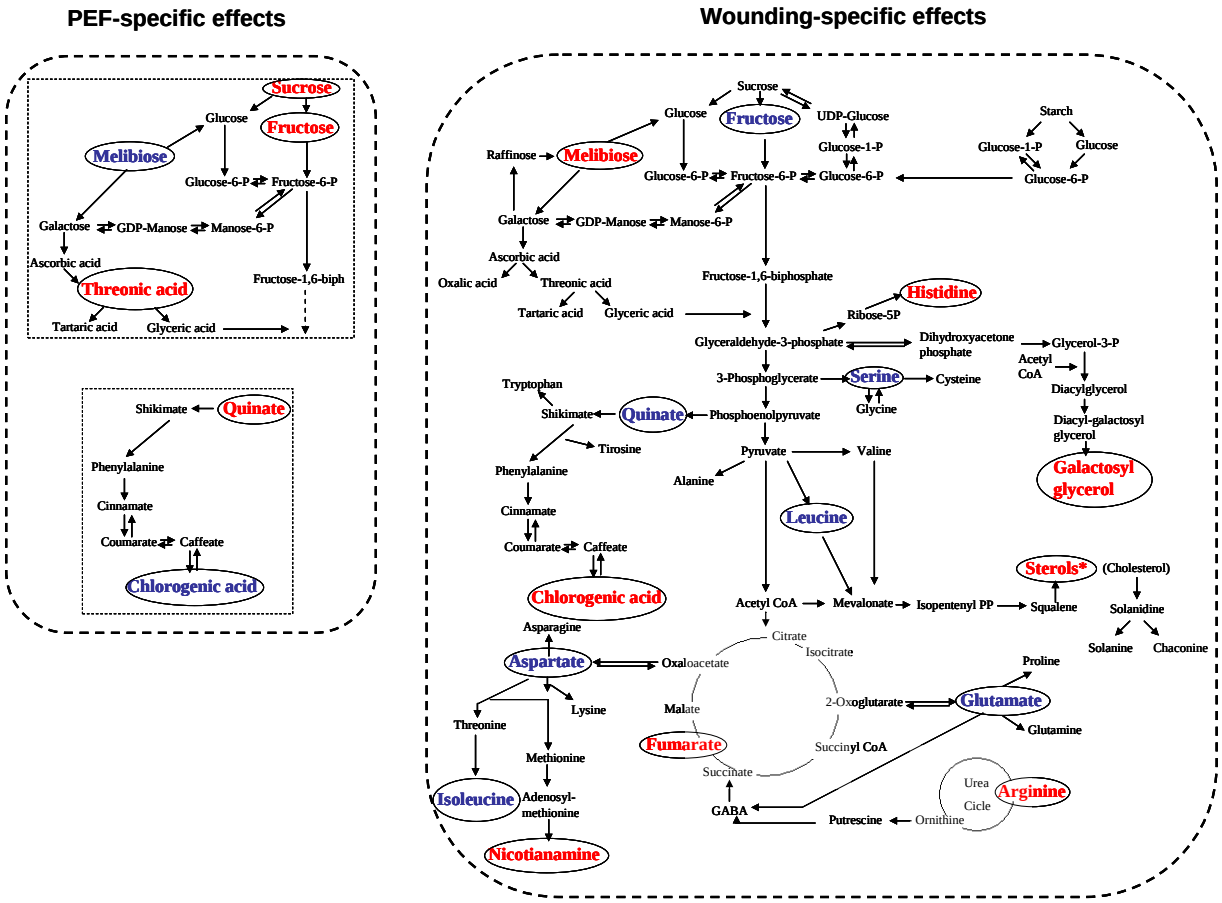

Fig. 3. *Changes in the levels of metabolites in potato tissue caused by wounding and by the application of PEF to the wounded tissue. The changes in metabolite contents, specific for each kind of stressor, are highlighted in the simplified metabolic maps with a circle. Metabolites are annotated red for increased concentration and blue for decreased concentration.*

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