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Metabolic profiling reveals a coordinated response of isolated lamb's (*Valerianella locusta*, L.) lettuce cells to sugar starvation and low oxygen stress

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ABSTRACT

Sugar starvation is a common phenomenon occurring in most leafy vegetables after harvest and storage. Additionally, leafy vegetables are subjected to low O_2 stress when stored in modified atmosphere conditions. In this study, the metabolism of isolated lamb's lettuce cells was studied upon sugar starvation under O_2 stress conditions, using ^{13}C labelled glucose. Fast depletions of the soluble sugars were observed, being more pronounced under aerobic conditions than under low O_2 stress conditions. Sugar starvation under aerobic conditions resulted in increased levels and decreased ^{13}C label incorporation of TCA cycle intermediates and amino and fatty acids originating from glycolytic and TCA cycle pathways, compared to starving cells incubated under low O_2 stress. On incubation under low O_2 stress a switch in metabolism from aerobic to fermentation metabolism was observed. Under low O_2 stress conditions, increased levels and ^{13}C label incorporated in hexose phosphates, pyruvate, lactate, GABA, alanine, together with increased levels of acetaldehyde, ethanol and ethyl acetate was observed indicating fermentative metabolism was triggered.

1. Introduction

The soluble sugars, glucose, fructose and sucrose, constitute the primary energy reserves for most leafy vegetables being metabolized through the respiratory metabolism, leading to carbohydrate starvation. Carbohydrate starvation is a common phenomenon studied in many plant systems including wheat leaves (Wittenbach et al., 1982), sycamore (Journet et al., 1986), tobacco (Moriyasu and Ohsumi, 1996), lupine (Morkunas et al., 2003), and maize root tips (Dieuaide-Noubhani et al., 1992; Brouquisse et al., 1991). Most of these studies used plant model systems such as cell cultures, callus tissue cultures, and isolated plant organs for the ease of manipulating the culture medium composition. Cell cultures showed clear metabolic responses when sugar was omitted from the nutrient medium (Journet et al., 1986; Dieuaide-Noubhani et al., 1992, 1997).

In plants, O_2 plays a major role as final electron acceptor in the respiratory metabolism (Babcock, 1999). While plants have the ability to photosynthetically produce O_2, they can experience low O_2 conditions both in the field and during postharvest (van Dongen et al., 2009). Some leafy vegetables like romaine lettuce (Martínez-Sánchez et al., 2011), butterhead lettuce (Varoquaux et al., 1996) and lamb's lettuce (Ferrante et al., 2009) are kept under low O_2 conditions to preserve quality, while prolonging their postharvest life. While lowering the O_2 partial pressure might have beneficial effects slowing down ageing and senescence, extreme anaerobic conditions may induce excessive fermentation and deterioration of the produce resulting in off-odours, off-flavours and de-greening (Mcdonald et al., 1990; Smyth et al., 1998).

Different 'omics' approaches have been used to study the metabolic response of different plant systems to low O_2 stress and sugar starvation (Hatoum et al., 2016; Lee et al., 2011; Lara et al., 2011; Pedreschi et al., 2009; van Dongen et al., 2009; Geigenberger et al., 2000; Ampofo-Asiama et al., 2014a). Because of the occurrence of cyclic and parallel pathways in plants, metabolite analysis should be performed by complementing data on metabolite levels with labelling data obtained through the use of isotope-labelled substrates (Wiechert, 2001). This is essential in

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providing insight in the regulation and control of metabolic pathways when plant systems are challenged by stress conditions. By combining both metabolite and labelling data, the metabolic response of isolated lamb's lettuce cells at different temperatures could be studied. In that study, sugar starvation was more pronounced at high temperatures than at low temperatures (Mbong et al., 2016). With the labelling data, a switch of substrate from sugars to protein and lipids breakdown could be observed. Since leafy vegetables are stored under MAP in addition to conditions where there is limited or no photosynthetic active radiation, studying this combined effects of low O_2 stress and sugar starvation would increase our understanding of how they adapt under theses stress conditions.

Therefore, the objective of this work was to study the metabolic changes occurring in leafy vegetables exposed to conditions that induce sugar starvation in combination with low O_2 stress using isolated lamb's lettuce cells as a model system. By using GC–MS as analytical platform, we complemented metabolome data with ^{13}C labeling incorporation by pre-labelling the isolated cells with uniformly labelled [U-$^{13}C_6$] glucose, followed by the induction of sugar starvation under normoxic (21 kPa O_2), hypoxic (1 kPa O_2) and anoxic (0 kPa O_2) at 18 °C.

2. Materials and methods

2.1. Plant material

Lamb's lettuce (*Valerianella locusta*, L. var. 'Gala'), grown at a 12 h light/12 h dark regime at 150 μE m^{-2} s^{-1}, was harvested early in the morning at commercial harvest stage from a commercial grower (Duffel, Belgium). Prior to transportation to the lab, the lettuce was washed with chlorinated distilled water to remove soil debris. Upon arrival, the harvested lettuce was cleaned with 0.0005% (v/v) NaOCl solution, rinsed 5 times with deionized water and dried using paper towel.

2.2. Reagents

The following reagents were used for the preparation of the cells suspensions: polyethylene glycol (PEG of average M = 3350 g/mol; Sigma Aldrich, cat. no. 202444), glucose hydrate (Vel, cat. no. 5974), 4-morpholineethanesulfonic acid hydrate (MES, pH 5.8; Sigma Aldrich, cat. no. M8250), magnesium sulphate (Sigma Aldrich, cat. no. D9434), calcium chloride (Sigma Aldrich, cat. no. C5670), bovine serum albumin (BSA, Sigma Aldrich, cat. no. A7906), and pectinase from *Aspergillus niger* (Sigma Aldrich, cat. no. P4716), and [U-$^{13}C_6$] Glucose (Sigma, cat. no. 389374).

2.3. Leaf sap osmolality measurement

Leaf sap osmolality measurement was performed as described in Mbong et al. (2016).

2.4. Lamb's lettuce cells isolation and cell viability

The procedure for lamb's lettuce cells isolation and viability testing is as described in Mbong et al. (2016).

2.5. Sugar starvation experiment

To study the effect of sugar starvation at various O_2 conditions, the isolated cells in glucose-free PEG suspension were transferred to a Lambda Minifor bench-top laboratory bioreactor (Lambda Laboratory Instruments, Czech Republic) in the dark. The experiment was divided into two phases. In the first loading phase, the isolated cell suspension was incubated with 20 mM uniformly ^{13}C [U-$^{13}C_6$] labelled glucose to a final volume of 250 mL, giving a cell to medium ratio of 3:1. The pH and temperature of the medium were maintained at 5.8 and 18 °C, respectively. Dissolved gas conditions were controlled by continuously bubbling ambient air through the medium at a rate of 10 L h^{-1}. The cells were first allowed an adaptation period of 1 h in the bioreactor before the initial sample was taken (0 h sampling time point). Additional samples were taken after 2, 6, 12 and 24 h. Sampling was carried out by withdrawing 15 mL of medium containing cells from the bioreactor, washing with glucose free medium, snap freezing in liquid nitrogen and storing at − 80 °C. A similar [U-$^{13}C_6$] glucose loading phase at ambient air was performed for all 3 different O_2 levels studied in this work.

After 24 h the second (starvation) phase was initiated by washing the cells by repeatedly replacing the medium by fresh glucose-free PEG solution during a 1 h period. The washing medium was pre-flushed using air with either 21 kPa, 1 kPa or 0 kPa of O_2. After replacing the medium, the cells were continuously flushed using air with either 21 kPa, 1 kPa or 0 kPa of O_2, under the same conditions of pH, temperature and gas flow rate as during the loading phase. Subsequent samples were taken 26, 32, 38, 50, 74, 98, 122, 146 and 170 h after the start of the experiment. All experiments were carried out in the dark with three biologically independent replicate experiments per condition.

2.6. Respiration measurements for unstarved and starved cells

To select the O_2 conditions for this study, respiration measurements of both the unstarved and starved cells were conducted using a biological O_2 monitor working on Clark's O_2 electrode principle following the procedure from Lammertyn et al. (2001) and Ampofo-Asiama et al. (2014a). A volume of 4 mL cell suspension with a known concentration of cells was introduced in the cuvette and flushed with normal air to obtain the same initial concentration of O_2. The depletion of O_2 was monitored over time and the maximum O_2 uptake rate and K_m of respiration of the unstarved and starved cells were estimated by non-linear least squares optimization using the public domain OptiPa software (Hertog et al., 2007).

2.7. Analysis of fermentation metabolites (ethanol, ethyl acetate, acetaldehyde)

Fermentation metabolites were measured both in the cell suspension and in the gas stream passing through the suspension. To this end 5 mL of cell suspension was transferred to a 20 mL headspace glass vials, capped, snap frozen in liquid nitrogen and subsequently heated to 65 °C in a water bath for 20 min. At the same time, gas samples were obtained from the outlet of the bioreactor using 1 L vacuum sample bags (TEDLAR, SKC Inc., USA). Ethanol, ethyl acetate and acetaldehyde in both the sample bags and headspace vials were measured using a compact gas chromatograph (Interscience, B.V., The Netherlands). The compact GC was equipped with a 15 m long MXT-wax column with 0.53 mm internal diameter and 0.5 μm stationary phase. The metabolites were detected with a flame ionization detector (300 μL min^{-1} air and 30 μL min^{-1} H_2) using helium as a carrier gas (30 μL min^{-1}). Calibration curves were used to determine the absolute concentration of each fermentation metabolite. The production rate were calculated as the sum of the ethanol, acetaldehyde and ethyl acetate in both the headspace vials and the vacuum bags.

2.8. Analysis of primary metabolites

The extraction and derivatization of primary metabolites prior to GC-Ms analysis was performed following the procedure of Mbong et al. (2016).