Postharvest Biology and Technology 125 (2017) 1–12

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Research paper

The effect of temperature on the metabolic response of lamb's lettuce (*Valerianella locusta*, (L), Laterr.) cells to sugar starvation

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ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 26 September 2016
Accepted 27 October 2016
Available online xxx

Keywords:
Isolated lamb's lettuce cells
Sugar starvation
Temperature
Metabolome
^{13}C label

ABSTRACT

Fresh leafy vegetables are highly perishable and may suffer from sugar starvation during postharvest storage. To fully understand their metabolic response to sugar starvation, isolated lamb's lettuce (*Valerianella locusta*, (L) Laterr.) cells were used as a model system to study biochemical and metabolic stress response to sugar starvation at 1 °C, 18 °C and 25 °C. The effect of sugar starvation was minimal at 1 °C. While the higher temperature showed clear impact of sugar starvation on the overall metabolic profile no significant differences were observed between the starvation at either 18 °C or 25 °C for the main sugars (glucose, glucose-6-phosphate, fructose, fructose-6-phosphate and sucrose). Biochemical and metabolic changes of the isolated cells upon sugar starvation involved a decrease in the levels of sugars, except for trehalose and ribose, as well as an increase in the levels of sugar alcohols. Sugar starvation altered the central metabolism by decreasing the levels of the intermediates of the glycolytic pathway, except for 3-phosphoglycerate and pyruvate. Increased levels of the intermediates of the tricarboxylic acid cycle were also observed. ^{13}C labelling data showed a decreased label accumulation in almost all metabolites, except for mannitol, myo-inositol, and trehalose. The increase in the levels of free soluble amino and fatty acids with a corresponding decrease in their ^{13}C label suggested a breakdown of protein and triacylglycerides.

1. Introduction

Leafy vegetables, like lamb's lettuce (Enninghorst and Lippert, 2003), Chinese cabbage (Klieber et al., 2002), butterhead lettuce (Varoquaux et al., 1996), and crisphead lettuce (Fouse and Lipton, 1985), have been shown to have no significant pool of soluble carbohydrates or other respiratory substrates. The photosynthetic carbohydrates gathered before harvest make up a limited source of respiratory substrates. Given harvested leafy vegetables are stored in the absence of photosynthetically active radiation they may be subject to sugar starvation (Lipton, 1987).

Low temperature storage preserves postharvest quality by slowing down metabolic processes. The optimal storage range for leafy vegetables is 0–4 °C (Kader, 2002; Jacxsens et al., 2000, 2002). Since the storage life of these products is defined by the levels of their soluble photosynthetic sugars (such as, glucose, fructose and sucrose) at harvest (Enninghorst and Lippert, 2003), poorly controlled storage temperature could result in increased utilization of these soluble sugars resulting in sugar starvation. This can result in high economic losses. Notwithstanding the available literature on sugar starvation in plants, there is limited knowledge on the effects of sugar starvation on the metabolic response of fresh leafy vegetables during postharvest storages.

In plants, the induction of sugar starvation has shown to result in changes in their metabolism as an adaptation response to cope with this nutrient stress through catalytic processes such as protein and lipid. For example, *Arabidopsis* (Thimm et al., 2004), cells in maize root tips (Brouquisse et al., 1991; Dieuaide et al., 1992; Dieuaide-Noubhani et al., 1997), cultured suspension cells (Chen et al., 1994a, 1994b; Journet et al., 1986) and callus cells (Tassi et al., 1992), have been shown to modify their metabolism to survive when sugar was absent. These catabolic processes could be used to provide alternatives to sugar to sustain respiration and metabolic processes (Brouquisse et al., 1991; Chen et al., 1994a).

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http://dx.doi.org/10.1016/j.postharvbio.2016.10.013
0925-5214/

Metabolic profiling of the metabolic effect of sugar starvation on different plant cells has been carried out, but mostly without ^{13}C labelled substrate feeding. It is however important to note that since the actual activity of the metabolic pathways cannot be explained with metabolite levels alone, isotope-labelled substrates has proven to be essential in fully understanding the control and regulation of metabolic networks (Ratcliffe and Shachar-Hill, 2006; Schauer et al., 2005; Sauer, 2006). To realize extensive metabolite labelling, uniformly labelled stable isotope tracers are generally being used (O'Grady et al., 2012; António et al., 2015). Gas chromatography–mass spectrometry analysis (GC–MS) can be used to evaluate ^{13}C enrichment and isotopomer composition. Dieuaide-Noubhani et al., (1997) fed maize root tips with [1-^{13}C] glucose achieving close to steady-state labelling of carbohydrates and metabolic intermediates, but lipids and proteins were only scarcely labelled. Because of the large size of the intracellular pool of sugars and other polymers in vegetables, the incorporation and quantification of ^{13}C label can be a non-trivial task often requiring extended feeding times (Alonso et al., 2007). By analysing metabolic responses of fresh leafy vegetables exposed to sugar starvation in the presence of ^{13}C label, it becomes possible to study the regulation of metabolic networks by observing which fluxes through pathways are up- or down-regulated. By monitoring the metabolite changes as a function of time during dynamic labelling experiments, the metabolic flexibility of plant cells during sugar starvation can be studied in full detail. This study focused on fresh leafy vegetable cells as a model system as they can be more easily manipulated to introduce ^{13}C labelled substrate (glucose) as compared to the intact system.

The objective of this work was to study the metabolic and biochemical changes occurring in fresh leafy vegetables during sugar starvation using isolated cells as a model system. To this end, a protocol to isolate viable lamb's lettuce (*Valerianella locusta,* (L) Laterr.) cells from intact leaves were optimized. The extent of the metabolic changes induced by sugar starvation was evaluated as a function of different temperatures. Metabolic profiling data was complemented by ^{13}C labelling information by feeding isolated cells with uniformly labelled [U-^{13}C] glucose in the dark, followed by sugar starvation induced at either 25 °C, 18 °C or 1 °C. The intermediates of the central carbon metabolism and the free soluble amino, fatty and organic acids, together with their ^{13}C-label incorporation were quantified using GC–MS analysis.

2. Materials and methods

2.1. Plant material

Lamb's lettuce (*Valerianella locusta,* (L) Laterr., 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 from a commercial greenhouse and transported to the laboratory. Prior to transportation, the lamb's lettuce was washed with chlorinated distilled water to remove soil debris. Upon arrival in the laboratory, the harvested lamb's lettuce was cleaned with 0.0005% NaOCl, rinsed 5 times with deionized water and then dried.

2.2. Reagents

The following reagents were used: polyethylene glycol (PEG of average molecular weight of 3350; 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), [U-$^{13}C_6$] Glucose (Sigma, cat. no. 389374).

2.3. Leaf sap osmolality measurement

Prior to lamb's lettuce cells isolation, leaf sap osmolality was determined on mature leaves frozen in liquid nitrogen and ground to a fine powder. After thawing and centrifugation at 15,000 m/s for 20 min at 4 °C, the supernatant was collected, filtered and immediately used for water activity determination using a water activity meter (aw SPRINT, TH-500, Novasina, Switzerland). The osmolality of the cell sap was calculated from calibration curves using known osmolality of sodium chloride solutions. The osmolality calculated was used to prepare the suspension medium for the cells isolation and subsequent sugar starvation experiments.

2.4. Lamb's lettuce cells isolation and cell viability

Lamb's lettuce cells were isolated from commercially mature plants with fully-developed leaves. A maceration solution of 90 mM glucose, 7% (w/v) PEG, 15 mM MES buffer pH 5.8, 2 mM $MgSO_4$ and 0.4% (w/v) pectinase was prepared incubated at 55 °C for 10 min and put on ice for 10 min to cool to room temperature. Next, 3 mM $CaCl_2$ and 0.2% (w/v) BSA were added to the maceration solution. The maceration solution was then filtered using a 0.45 μm filter. About 30 striped leaves were then added to 50 mL of the maceration solution and vacuum infiltrated for 10 min in the dark. Finally, the leaves were incubated in the maceration solution in the dark for an additional 2.5 h at 18 °C while flushed with air (21 kPa O_2, 0 kPa CO_2, at 10 L/h). After the isolation process, an equal amount of isotonic glucose-free PEG solution (12% (w/v) PEG 3350, 15 mM MES buffer pH 5.8, 2 mM $MgSO_4$, 3 mM $CaCl_2$ and 0.2% (w/v) BSA) was added and the resulting solution was filtered through a nylon mesh (pore size 35–75 μm) in falcon tubes and the filtrate was decanted after a few minutes. The process of decanting was repeated five times. Dead and live cells were counted by the Evans Blue Exclusion staining technique (Shipway and Bramlage, 1973; Pushmann and Romani, 1983). The cell suspension (200 μL) was stained with 0.5% (w/v) Evans Blue for 5 mins at room temperature (20 °C) which stains the dead cells. The percentages of live and dead cells were estimated by counting on a haemocytometer under a light microscope (BX40-Olympus, Japan).

2.5. Sugar starvation experiment

To study the effect of different temperatures on sugar starvation, the concentrated 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 temperatures selected for this study were 1 °C, 18 °C (control), and 25 °C. The sugar starvation experiment was divided into two phase. In the first loading phase, the concentrated isolated cell suspension was incubated with 20 mM [U-$^{13}C_6$] 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 kept constant at 5.8 and 18 °C, respectively. Dissolved gas conditions were controlled by bubbling normal air through the medium at a rate of 10 L h^{-1}. The [U-$^{13}C_6$] glucose loading phase of the cells was the same for all 3 different temperature studies to ensure rapid incorporation of the labelled glucose. Immediately after the cells, together with [U-$^{13}C_6$] glucose were put in the bioreactor, sampling was started (0 h sampling time point). Samples were taken at 1, 2, 6, 12 and 24 h from the start of the