Journal of Chromatography B, 1000 (2015) 1–13

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantitative profiling of polar primary metabolites of two chickpea cultivars with contrasting responses to salinity

Daniel Anthony Dias[a,1], Camilla Beate Hill[b,*,1], Nirupama Samanmalie Jayasinghe[a], Judith Atieno[c], Tim Sutton[c,d], Ute Roessner[a,b]

[a] Metabolomics Australia, School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[b] School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[c] Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, The University of Adelaide, Glen Osmond, South Australia, 5064, Australia
[d] South Australian Research and Development Institute, GPO Box 397 Adelaide, South Australia 5001, Australia

ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 28 June 2015
Accepted 2 July 2015
Available online 13 July 2015

Keywords:
Quantitative profiling
Primary metabolites
GC–*QqQ*–MS
LC–*QqQ*–MS
Chickpea
Salinity

ABSTRACT

This study reports a GC–*QqQ*–MS method for the quantification of forty-eight primary metabolites from four major classes (sugars, sugar acids, sugar phosphates, and organic acids) which can be applied to a number of biological systems. The method was validated in terms of linearity, reproducibility and recovery, using both calibration standards and real samples. Additionally, twenty-eight biogenic amines and amino acids were quantified using an established LC–*QqQ*–MS method. Both GC–*QqQ*–MS and LC–*QqQ*–MS quantitative methods were applied to plant extracts from flower and pod tissue of two chickpea (*Cicer arietinum* L.) cultivars differing in their ability to tolerate salinity, which were grown under control and salt-treated conditions. Statistical analysis was applied to the data sets using the absolute concentrations of metabolites to investigate the differences in metabolite profiles between the different cultivars, plant tissues, and treatments. The method is a significant improvement of present methodology for quantitative GC–MS metabolite profiling of organic acids and sugars, and provides new insights of chickpea metabolic responses to salinity stress. It is applicable to the analysis of dynamic changes in endogenous concentrations of polar primary metabolites to study metabolic responses to environmental stresses in complex biological tissues.

1. Introduction

Salinity and other environmental stresses directly affect the normal growth, development, and reproduction of a plant, and therefore the primary metabolites involved in these processes. The diversity and fluctuation in biological stresses faced by a plant has led to adaptation through biochemical defense mechanisms including both primary and secondary metabolites to directly manage environmental perturbations. With the development of specialized protocols, targeted analysis of primary metabolites can provide a substantial amount of information to investigate complex changes in metabolism caused by different genotypic and/or environmental perturbations.

Chickpea (*Cicer arietinum* L.) is one of the world's most important pulse crops and ranks third in the world for food legume production [1]. Chickpea plants suffer damage even on moderately saline soils that have little impact on bread wheat, which in turn impacts on potential yields of chickpea in rotation with wheat on areas with sub-soil salinity. The reproductive phase is known to be even more sensitive to NaCl exposure than vegetative growth and germination [2], the early development of flower meristems, the conversion of flowers to pods, and the development of seeds in the pods are particularly susceptible to salinity stress. The number of flowers, pods, and seeds is significantly decreased in salt sensitive cultivars compared to tolerant chickpea lines upon salinity treatment [3], and carbohydrate supply to reproductive structures, such as the developing embryo is believed to be a limitation.

Abbreviations: DAS, days after sowing; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; CE, collision energy; EI, electron ionization; ESI, electrospray ionization; GC–*QqQ*–MS, gas chromatography-triple quadrupole-mass spectrometry; ISTD, internal standard; LC–*QqQ*–MS, liquid chromatography–triple quadrupole-mass spectrometry; LOQ, limit of quantification; MRM, multiple reaction monitoring; *m*/*z*, mass-to-charge ratio; Pro, proline; PBQC, pooled biological quality control sample; QC, quality control calibration standard mix; RI, retention time index; R^2, linear correlation coefficient; SRM, selected reaction monitoring.

* Corresponding Author.
E-mail address: camilla.hill@unimelb.edu.au (C.B. Hill).
[1] Equal first authors (D.A.D. and C.B.H.).

http://dx.doi.org/10.1016/j.jchromb.2015.07.002
1570-0232/

Plants have developed an extraordinary genetic diversity controlling the synthesis and regulation of metabolites that has largely been unexplored in the grain legumes. Data based on transcriptomics and proteomics analyses are insufficient to provide an understanding of all aspects of biological processes in response to abiotic stress, since those are ultimately mediated by metabolites. For example, changes in transcript or protein levels do not always correlate to actual changes of cell metabolites due to post-transcriptional and post-translational modifications that modulate protein activities [4]. However, only a few studies on primary metabolism in legumes are available, and these studies cover the model legumes *Medicago truncatula* [5], both model and cultivated legume species of the *Lotus genus* [6,7] and soybean [8].

Metabolomics is now being explored as a possible solution to these problems because it can capture the "ultimate phenotype" of such gene networks and their complex interaction with the environment [9]. Primary metabolites are mostly hydrophilic molecules directly involved in all biochemical processes, including growth, development, and reproduction. Compounds present in carbohydrate metabolism can be challenging to analyze due to the high diversity of compounds with a diverse array of functionalities (including neutral carbohydrates such as saccharides and polyalcohols, polysaccharides, and sugar acids), the often very similar fragmentation of isomers, and the co-elution of two or more compounds with very similar retention times which give rise to complex chromatograms. More effective tools and methods are required for efficient identification and quantification of compound classes, i.e., sugars and organic acids.

Liquid chromatography (LC) coupled to triple quadrupole (*QqQ*)–MS systems have benefited greatly from the high sensitivity and selectivity of tandem MS in the selected reaction monitoring (SRM) mode. Although the coupling of gas-chromatography (GC) to electron impact ionization (EI) mass spectrometry (MS) is one of most well-known and established techniques in analytical chemistry and one of the most developed instrument platforms for metabolite analysis [9]. GC-based methods have suffered a notable delay in the wide acceptance of the *QqQ* analyzer in comparison to LC–MS/MS. GC–MS has been widely used in metabolomics since it has significant separating power, is reproducible, easy to establish and requires a relatively low capital investment compared to other analytical technologies. GC–MS is an ideal analytical technology for the analysis of volatile compounds however most metabolites are not volatile and therefore need to be chemically derivatized in order to make them amenable for GC–MS.

In recent years, there has been a strong emphasis within the metabolomics community that quantitative data is important for biological studies since they describe accurately the actual concentration of the metabolites of interest. New *QqQ* instrumentation allows for higher selectivity and sensitivity and minimizes chromatographic interferences and is typically operated in multiple reactions monitoring (MRM) mode in which collision energies, dwell times and resolution parameters for each individual target compound is optimized using authentic standards, thus, enhancing sensitivity and selectivity [10]. In a single chromatographic run, the application of MRM can simultaneously monitor a large number of MS–MS transitions.

Metabolomics aims to provide a comprehensive and unbiased analysis of all metabolites with a low molecular weight present in a biological sample [4,9]. Due to the structural diversity of metabolites, there is currently no single methodology that can detect the complete metabolome, which is why several extraction methods and instrument platforms are established to analyze highly complex mixtures. Here we used both GC–MS and LC–MS techniques as they are complementary to each other: to accurately quantify primary metabolites of carbon and nitrogen metabolism, GC–MS-based metabolite analysis of organic acids, sugars, sugar alcohols, and sugar acids was carried out on a GC–*QqQ*–MS (Agilent 7890 GC coupled to 7000 Triple quadrupole MS). We have demonstrated the applicability of the method specifically to the extraction of metabolites of flower and pod tissue of two chickpea cultivars, 'Genesis 836' and 'Rupali', before and after salinity stress. To investigate the effects of salinity on other metabolite classes of primary metabolism, we have combined it with an established LC–MS-based metabolomics method for quantification of amine-containing metabolites carried out on a LC–*QqQ*–MS (Agilent 1290 LC coupled to 6490 triple quadrupole MS) according to the standardized protocol developed by [11].

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents were purchased from Sigma–Aldrich (Australia) and were either of analytical or mass spectrometric grades. Deionized water (18.2 MΩ) was produced using a Synergy UV Millipore System (Millipore) was used throughout.

2.2. Plant growth and harvest

The desi chickpea cultivars used in the experiment were Genesis 836 (salt tolerant) and Rupali (salt sensitive). Genesis 836 is a direct introduction from the International Centre for Research in the Semi-Arid Tropics (ICRISAT, Syria), while Rupali was bred by the Department of Agriculture, Western Australia (DAWA), and the Centre for Legumes in Mediterranean Agriculture (CLIMA), The University of Western Australia.

The experiment was conducted in a glasshouse at the University of Adelaide Plant Accelerator facility (Waite Campus, South Australia). Temperature and humidity were controlled and ranged from 24 ± 2 °C and 40% (day), and 18 ± 2 °C and 90% (night), respectively.

Five seeds of each of the cultivars were sown 2 cm deep in pots (19.46 cm height × 14.94 cm diameter) filled with 2.5 kg of 50% University of California (UC) mixture (1:1 peat:sand) and 50% cocopeat (pH 7.5; electrical conductivity ($EC_{1:5}$ 603 μs/cm)). The soil was inoculated with *Rhizobium* inoculum (Group N) prior to sowing. Prior to salt application, plants in each pot were thinned to two uniform plants. At flowering, 21 and 25 days after sowing (DAS) for Rupali and Genesis 836 respectively, each pot received either 0 or 60 mM NaCl (1.3149 g NaCl pot^{-1}) equivalent to applying 100 ml of 0 mM NaCl (untreated pots) or 225 mM NaCl (treated pots) delivered in two increments through the base of the pots by standing the pots in saucers containing saline solution. Each treatment was replicated four times and randomized in a randomized complete block design (RCBD). The pots were watered every two days and maintained at field capacity, 15% (w/w)-determined gravimetrically to maintain salt concentration in the pots and to also avoid salt leaching out of the pots as a result of over watering.

Flowers and pods were harvested and pooled from two plants in each pot 31 and 48 DAS (for cv. Rupali) or 35 and 52 DAS (for cv. Genesis 836). The samples were immediately frozen in liquid nitrogen, and thereafter stored at −80 °C.

2.3. Plant sample extraction and preparation

A modified method for the preparation of plant extracts was used as described previously by [24]. For each chickpea cultivar, approximately 30 mg of frozen flower and pod tissues was weighed into cryomill tubes (Precellys lysing kit, Bertin Technologies). Subsequently, 400 μL of 100% methanol containing 4% internal standard (from a stock solution containing 0.5 mg mL^{-1} $^{13}C_6$-sorbitol and 0.5 mg mL $^{13}C_5$–^{15}N valine) was added to the samples,