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SHORT COMMUNICATION

Characterization of the primary metabolome during the long-term response to $NaHCO_3$-derived alkalinity in *Lotus japonicus* ecotypes Gifu B-129 and Miyakojima MG-20

Cesar D. Bordenave[1] · Rubén Rocco[1] · María Florencia Babuin[1] · María Paula Campestre[1] · Francisco J. Escaray[1] · Andrés Gárriz[1] · Cristian Antonelli[1] · Pedro Carrasco[2] · Oscar A. Ruiz[1] · Ana B. Menéndez[3,4]

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Abstract This study compares the response of two ecotypes of the model species *Lotus japonicas*, MG-20 and Gifu-B-129, to soil alkalinity, in terms of plant survival and changes in global primary metabolome profiles. After 54 days of treatment with 30 mM $NaHCO_3$, a higher survival was registered in MG-20, with respect to Gifu-B-129 plants. Gas chromatography–mass spectrometry (GC–MS) analysis of shoot extracts from both ecotypes yielded 123 different analytes, 62 of which were identified, including organic acids (OA), amino acids (AA), sugars and polyols. Glycolysis, TCA cycle and amino acids metabolism pathways were differently affected by alkalinity according to the ecotype. The lower tolerance of Gifu B-129 plants to 10 mM $NaHCO_3$, compared with MG-20 ones could be related, at least partially, to the differential accumulation of phosphoric, lactic, threonic, succinic and *p*-coumaric acids, as well as β-alanine and valine.

Keywords Alkalinity · *Lotus japonicus* · Gifu B-120 · Miyakojima MG-20 · Metabome · GC–MS

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C. D. Bordenave and R. Rocco contributed equally to this work and, therefore, should be regarded both as first authors.
A. B. Menéndez and O. A. Ruiz shared the same responsibility on this work and, therefore, should be regarded both as senior authors.

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✉ Ana B. Menéndez
anamen@bg.fcen.uba.ar

[1] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús/Universidad Nacional de General San Martín-Consejo Nacional de Investigaciones Científicas y Técnicas (IIB-INTECH/UNSAM-CONICET), Chascomús, Argentina

[2] Departamento de Bioquímica y Biología Vegetal-Universitat de Valencia, Valencia, Spain

[3] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[4] INMIBO (CONICET), Buenos Aires, Argentina

Introduction

Soils containing elevated Na_2CO_3 and $NaHCO_3$ concentrations are characterized by high pH, poor fertility, dispersed physical properties, low water infiltration capacity and high bicarbonate levels (Marschner 1995). In the soil buffer, bicarbonate (HCO_3^-) reduces Fe and P uptake by plants, leading to leaf chlorosis (Valdez-Aguilar and Reed 2010) and stunting (Bie et al. 2004). The ability of plants to overcome unfavorable environmental conditions involves transcriptomic and proteomic changes, which ultimately affect metabolism and the metabolite balance of cells.

Gas chromatography coupled to mass spectrometry (GC–MS) together with bioinformatic tools allows the robust, reliable, sensitive and fast monitoring of simultaneous changes of hundreds of metabolites (Fiehn et al. 2000; Roessner et al. 2001; Desbrosses et al. 2005; Lisec et al. 2006; Kopka et al. 2005). In addition, an open access metabolome database was presented, including custom mass spectral libraries, metabolite profiling experiments and additional information, which constitutes an exchange platform for experimental research activities (Kopka et al. 2005). Metabolic profiles analyses were previously performed to study the effect of alkalinity in the dicot halophyte *S. corniculata* (Pang et al. 2016) and in three

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poaceous (Gramineae) species: *Oriza sativa* (Rose et al. 2012), *Poa pratensis* (Hu et al. 2015) and *Triticum aestivum* (Guo et al. 2015). However, at the best of our knowledge, no metabolomic study has been undertaken so far describing metabolites responses to alkalinity in legumes species.

Lotus L. (Loteae, Fabaceae) is a cosmopolitan genus comprising several species acknowledged by their elevated adaptability to diverse soil constrains. These species are used as an alternative forage in South America and Australia, and as cover crops for dunes revegetation and reclamation of heavy metal-contaminated or burned soils in Europe (Escaray et al. 2012). In addition, the species *L. japonicus* has become a model for legumes with determinate nodulation type due to its characteristic genome features, and the development of a variety of resources for functional genomics, which has helped in the advance of legume research.

Comparisons between genotypes with contrasting tolerance to abiotic stress may improve understanding of stress-tolerance mechanisms and can be used in the discovery of stress-tolerance genes. Recently, our group showed that plants of the most widely used *L. japonicus* ecotypes, Gifu B-129 and Miyakojima MG-20 (from now on MG-20), decreased their Fe and Zn contents, and differed in the expression pattern of genes that could play important roles in the generation of a new Fe/Zn homeostatic condition, during their response to 10 mM $NaHCO_3$ (Babuin et al. 2014).

In this work, we hypothesize that Gifu B-129 and MG-20 also diverge in their metabolomic response to alkalinity.

The aim of this study was to compare the response of these two *L. japonicus* ecotypes to soil alkalinity in terms of plant survival and changes in global primary metabolome profiles.

Materials and methods

Plant material and growth conditions

Seeds from *L. japonicus* ecotypes MG-20 and Gifu B-129 were scarified with concentrated sulfuric acid (98%) 3 min, washed ten times with sterile distilled water and sown in Petri dishes containing water-agar (0.8%). Plates were incubated for 7 days in a growth chamber, with a 16/8 h photoperiod at 24/21 °C ± 2 °C (day/night) and 55/65 ± 5% relative humidity. Light intensity (250 µmol m^{-2} s^{-1}) was provided by Grolux fluorescent lamps (F 40 W). One seedling was transferred to a 8 cm (diam) × 20 cm (length) cylindrical pot containing washed sand mix (50% fine/50% coarse sand; pH 7.0; E.C. = 0.05 mS cm^{-1}) and irrigated with 0.5 × Hoagland's nutrient solution (Hoagland and Arnon 1950). A drip irrigation system (9001 Digital Watering Timer Weekly Program, ELGO®, http://www.elgo.co.il; flow rate = 6.25 ml/h) was used according to Paz et al. (2012).

Experimental design

Experiments followed a completely randomized design. Two ecotypes were evaluated, *L. japonicus* MG-20 and *L. japonicus* Gifu B-129, under two treatments: a control treatment without bicarbonate and an alkaline treatment with bicarbonate addition. Three successive independent experiment were performed, each consisting of four sample sets (control/alkaline and Gifu B-129/MG-20). In every experiment, each sample set represented one replicate, which consisted of a pool from 24 independent plants. Each biological replicate was later sub-divided into 2–6 technical replicates for derivatization and GC–MS analysis.

Alkaline treatment

The tolerance of MG-20 and Gifu genotypes to alkalinity was compared on the base of their survival to 30 mM $NaHCO_3$, applied during 54 days and expressed as percentage of alive plants (total = 40 plants), in two independent experiments. For the metabolomic study, lkaline stress treatment was imposed during 21 days by adding 10 mM $NaHCO_3$ to the 0.5 × Hoagland's solution and was started when plants were at the two full developed leaves stage. Control treatment consisted of plants irrigated with 0.5 × Hoaglands solution without $NaHCO_3$. The pH and E.C. of irrigation solutions were maintained at pH/E.C. (mS cm^{-1}) 6.2/1.2 and 8.2/1.9, for control and alkaline treatments, respectively.

Gas chromatography–mass spectrometry (GC–MS)

Plants were harvested, divided into shoots and roots, frozen in liquid N_2 and stored at −80 °C until they were processed. Metabolites were extracted from shoot tissue based on a modified version of the method used in Lisec et al. (2006). Briefly, 1460 µl of 100% methanol and 60 µl of 0.2 mg ml^{-1} ribitol were added to 100 mg of the frozen ground tissue and incubated at 70 °C for 15 min. Samples were centrifuged for 10 min at 12,000 rpm and the supernatant transferred to a new tube, then 1500 µl of water and 750 µl $CHCl_3$ were added and the sample was thoroughly vortexed and centrifuged for 10 min at 4000 rpm. Then, 150 µl of the polar phase was transferred to a new tube for trymethylsilylation (TMS) derivatization. All aliquots for derivatization were dried under vacuum, filled with N_2 gas and stored on silica gel.

Derivatization

Dry residues were derivatized based on a modified version of the method used in Lisec et al. (2006). For this, dry residues were re-dissolved and derivatized (120 min, 37 °C) in 70 µl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine. The sample was then treated with 40 µl MSTFA (*N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide).

Analysis conditions

GC–MS analysis was performed with a mass spectrometer (5973N, Agilent Technologies, EEUU), and a 7683 autosampler (Agilent Technologies, Stockport, UK), coupled to a gas chromatograph (6890N, Agilent Technologies, EEUU) with a HP-5MS UI column (Agilent, 30 m × 0.25 mm, 0.25 µm film thickness). Samples (1 µl) were injected onto the GC column using the hot needle technique. Helium was used as the carrier. GC oven temperature was 70 °C for 5 min, increased to 310 °C at 6 °C min^{-1}, for 4 min, split flow was adjusted at 1 mL min^{-1}, and injector temperature was at 250 °C. Mass spectra were recorded at 70 eV, mass range of *m*/*z* 30–650.

Metabolites quantification

Metabolites were quantified based on a modified version of the method used in Colebatch et al. (2004). Peak areas corresponding to each metabolite were integrated using ChemStation Integrator Algorithm (Agilent ChemStation Software). The levels of the compounds were calculated as the relative response ratio of peak areas of different compounds related to the peak area of ribitol (which served as an internal standard), and normalized with respect to the dry weight of the sample (according to Nikiforova et al. 2003). Metabolites that could not be consistently detected in all technical replicates of at least one sample set were not further analyzed.

Metabolites identification

Metabolites were identified by computer matching of their mass spectra against commercial libraries (Nist, Nist_msms, mainlib, replib, wiley7n). Mass spectral matching was manually checked against the mass spectra collection of the Golm metabolome database (Kopka et al. 2005; Schauer et al. 2005).

Statistical analysis

Response ratios of metabolites levels were subjected to log10-transformation, and differences between control and alkaline situations were compared according to the Student's *t* test ($P < 0.05$). Analyses were performed with the InfoStat statistical package (Di Rienzo et al. 2010).

All chemicals were purchased from Sigma-Aldrich.

Results and discussion

Evaluation of alkalinity tolerance of Gifu B-129 and MG-20

When the stress treatment was performed with 30 mM $NaHCO_3$ during 54 days, a higher survival was recorded in the MG-20 (93.8%; SE = ±6.2), compared with the Gifu B-129 (42.50%; SE = ±17.50) ecotype, whereas 100% of control plants survived in both ecotypes. On this basis, it was concluded that Gifu B-129 was less tolerant to alkalinity than MG-20. In contrast, 10 mM $NaHCO_3$ (during 21 days) caused no plant death, allowing the analysis and comparison of the long-term effect caused by elevated pH on both *L. japonicus* ecotypes.

Metabolomic analysis

GC–MS analysis of shoot extracts from both ecotypes yielded 123 analytes, with 78 of them being consistently detected over all the replicates in at least one treatment. Sixty-two, out of these 78 analytes were successfully matched against libraries, allowing the identification of a variety of metabolites including organic acids (OA), amino acids (AA), sugars and polyols (Fig. 1; Supplementary material. Table 1). GC–MS-based analysis revealed that alkalinity affected metabolites profiles of both *L. japonicus* ecotypes, being 34.5% of the changes shared by the two ecotypes, and 63.5% of them occurring in a genotype-specific manner (Supplementary material. Table 1). As it was previously observed in metabolomic studies performed on wheat (Guo et al. 2015), bluegrass (Hu et al. 2015) and *S. corniculata* (Pang et al. 2016), alkalinity affected glycolysis, TCA cycle, and organic acids and amino acids metabolisms.

Metabolites affected by alkalinity in both ecotypes

The GC–MS analysis showed that citramalic and citric acids significantly increased due to alkalinization in both ecotypes. These increases were 54 and 34% higher in Gifu B-129 than in MG-20, for citramalic and citric acids, respectively. Increases of these OAs are typical responses to alkalinity in several plant species (Ding et al. 2014; Hu

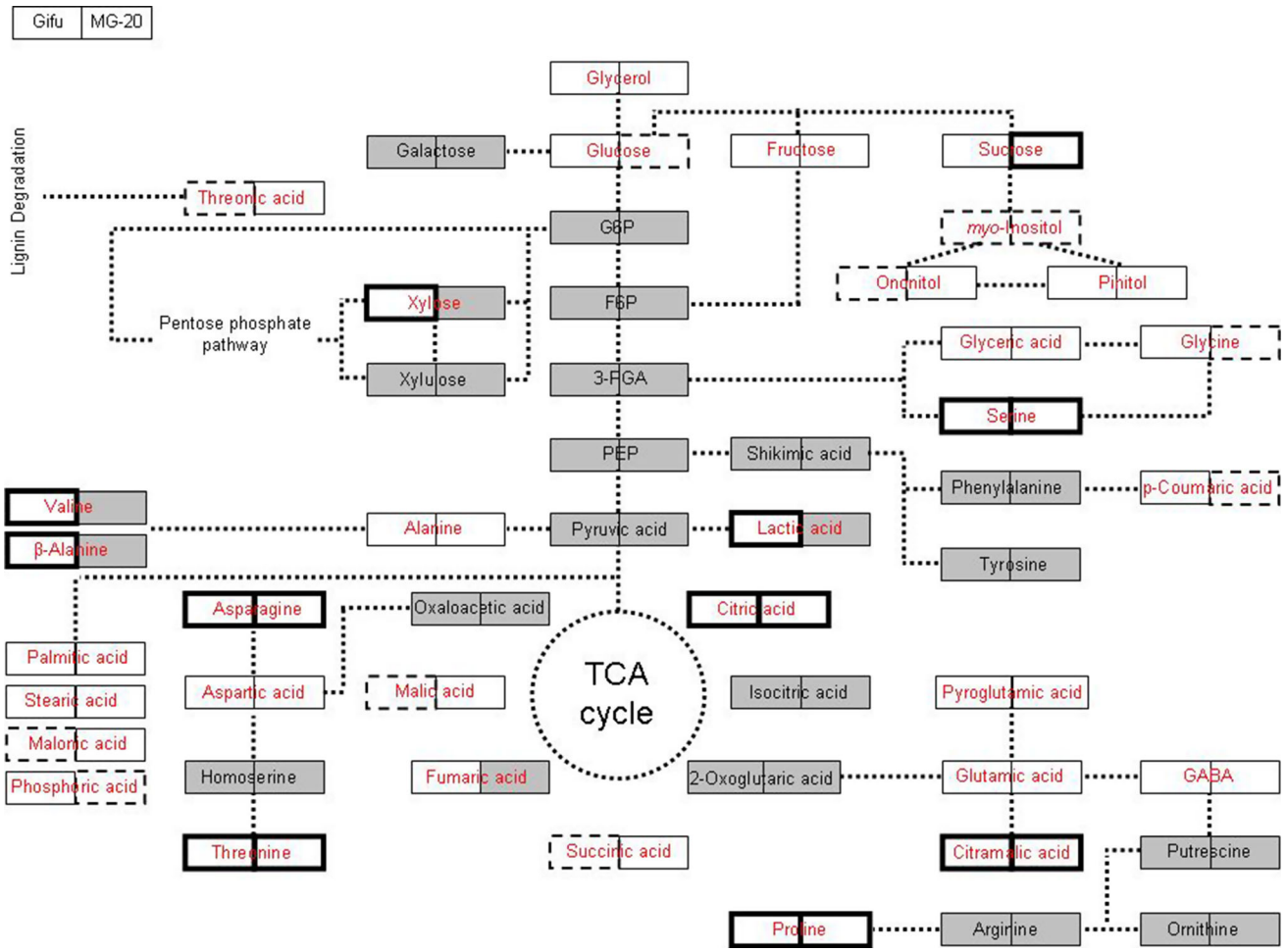

Fig. 1 Scheme of significant changes in metabolites abundance after alkaline treatment in Gifu B-129 (*right rectangles*) and MG-20 (*left rectangles*) shoots. *Arrows* indicate one or more steps pathways between two metabolites. *Dashed rectangle* frames indicate significantly decreased and *thickened frames* indicate significantly increased, whereas regular frame means unchanged metabolite level. *Grey color* indicates not detected metabolite in either treatment. Statistical significance was determined according to Student's *t* test ($P < 0.05$)

et al. 2015), and has been suggested that they may compensate for the Fe and P imbalance induced by alkalinity (Duda and Cherry 1971; Roschzttardtz et al. 2011; Rellán-Álvarez et al. 2010; Roschzttardtz et al. 2013; Pang et al. 2016). However, citrate also correlated positively with hydrogen peroxide concentrations and cell damage in rice (Rose et al. 2012), posing the question of whether citrate plays a role in alkali-stress alleviation or contributes with the stress to plant damage. The higher citrate response ratio found in the sensitive (Gifu B-129), compared with the tolerant (MG-20) ecotype, agrees with the view that this compound could increase plant damage, instead of improving plant nutrition.

Our results showed that asparagine, serine, proline and threonine were also increased by alkalinity in both ecotypes (Supplementary material. Table 1; Fig. 1). A recent microarray study performed on plants of the MG-20 and Gifu B-129 ecotypes subjected to alkalinity, showed that most of the regulated probe sets corresponding to genes coding for proteins involved in metabolism were related to protein degradation (Babuin et al. 2014). This result, along with the alkaline induction of a zinc metalloproteasa in *S. corniculata*, with functions in the efficient degradation of proteins (Pang et al. 2016), suggests that the increases in amino acids here observed could be originated, at least in part, from protein turnover. Asparagine was the most incremented AA in alkalinized plants of both ecotypes. Under high soil pH conditions, NO_3^- uptake is limited and NH_4^+ is the preferred plant nitrogen source (Rao and Rains 1976; Hageman 1984). Because NH_4^+ is toxic to plant tissues at relatively low levels, it is rapidly assimilated to asparagine in the roots, for its transport to the shoot (Heldt and Piechulla 2011). Taken together, this information suggests that de novo asparagine synthesis in roots, followed by its transport to the shoots via xylem, could have also contributed (besides protein turnover) with the

increase in pools of this amino acid detected in *L. japonicus* shoots. As increases of serine and proline have been previously observed in several *Lotus* species subjected to drought stress (Díaz et al. 2005), our results showing that serine and proline levels were increased by alkalinity in Gifu B-129 and MG-20 plants, suggest that, although the salt concentration was very low (10 mM), these plants could be subjected to osmotic, in addition to alkaline stress.

In addition, alkalinity-induced decreases of *myo*-inositol were registered in both ecotypes. This polyol has been involved in the tolerance to several abiotic stresses in diverse plant species (Taji et al. 2006; Iwata and Koizumi 2012; Kumar et al. 2013). In tobacco for example, the overproduction of ononitol (a *myo*-inositol derivative) led to increased salt and drought tolerance (Sheveleva et al. 1997). Thus, the strong *myo*-inositol reduction in alkalinized plants of both *L. japonicus* ecotypes here observed, could be regarded as one facet of the alkalinity-induced plant weakening.

In addition, this analysis revealed an unknown metabolite that matched the analyte identified as A159003 on the Golm Metabolome database increased on both alkalinized ecotypes, and a not yet identified metabolite (here referred as ui014) that were detected exclusively on alkalinized plants of Gifu B-129 and MG-20.

Metabolites affected by alkalinity in a genotype-specific manner

In Gifu B-129 plants sucrose was increased and glucose reduced, whereas MG-20 plants presented accumulated xylose and decreased ononitol. Phosphoric acid was detected in both ecotypes, being strongly reduced exclusively in Gifu B-129 plants and not significantly affected in MG-20 ones. Phosphoric acid is a component of numerous key organic metabolites such as nucleic acids, phospholipids and sugars. Therefore, its reduction, exclusively in alkalinized Gifu B-129 plants could be related to the fact that this ecotype resulted more affected by alkalinity than MG-20. In addition, there were reductions in threonic, malonic and succinic acids pools and accumulation of lactic acid in MG-20, but not in Gifu B-129 plants. *p*-Coumaric acid decreased only on alkalinized Gifu B-129 plants. This hydroxycinnamic acid plays a role as antioxidant (Shahidi and Chandrasekara 2010), and it is incorporated into newly forming lignin polymers (Hatfield and Marita 2010). Taken together, former results claim for further research, specifically addressing the compartmentalization of these metabolites within cell, to assess whether their patterns of alkalinity-induced variations may determine differences in cytosolic pH between both ecotypes.

The alkalinity-induced increase in sucrose observed in the Gifu B-129 ecotype, is also in line with results obtained in bluegrass (Hu et al. 2015) and wheat (Guo et al. 2015). Sucrose is the main transport sugar in most plant species. Thus, the concomitant glucose reduction observed in Gifu B-129 (and also in bluegrass), suggests that under alkalinity, plant privileges sugar transport over energy generation.

Another genotype-specific finding in our work was the occurrence of increased levels of valine and β-alanine exclusively in MG-20 plants. These metabolites could account for differences in tolerance between both ecotypes, since distinct functions in the tolerance to diverse plant stresses have been reported for several AA (Barnett and Naylor 1966; Greenway and Munns 1980; Gilbert et al. 1998; Szabados et al. 2011).

Finally, two unidentified metabolites accumulated on alkalinized MG-20 plants (ui009; ui013), whereas five unidentified metabolites (A216006; A217004; NA145015; ui007; ui016) modified their levels on MG-20, being three of them increased (Supplementary material Table 1).

As a whole, our results provide an overview of the metabolic syndrome of changes occurring in Gifu B-129 and MG-20 upon alkalinization.

Author contribution statement CDB, RR, MFB, CA and MPC contributed with the experimental work (set up, plant cultivation, statistical analysis), counting, and processing plant material for GC–MS, and results discussion. CDR, FJE and PC were in charge of spectra analysis, identification and quantification of metabolites. FJE, AG, PC, OAR and ABM participated of experimental design and results discussion. CDB, AG and ABM collaborated with manuscript writing.

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