

Characterization of the primary metabolome during the long-term response to NaHCO₃-derived alkalinity in *Lotus japonicus* ecotypes Gifu B-129 and Miyakojima MG-20

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Abstract This study compares the response of two ecotypes of the model species *Lotus japonicus*, 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 NaHCO₃, 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 NaHCO₃, 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-129 · Miyakojima MG-20 · Metabome · GC–MS

Introduction

Soils containing elevated Na₂CO₃ and NaHCO₃ 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 (HCO₃[−]) 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; Liseac 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 NaHCO₃ (Babuín 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 Gro-lux 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 experiments 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 NaHCO₃, applied during 54 days and expressed as percentage of alive plants (total = 40 plants), in two independent experiments. For the metabolomic study, alkaline stress treatment was imposed during 21 days by adding 10 mM NaHCO₃ 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 × Hoagland's solution without NaHCO₃. 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 N₂ 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 CHCl₃ 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 trimethylsilylation (TMS) derivatization. All aliquots for derivatization were dried under vacuum, filled with N₂ 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 μ l of 20 mg ml⁻¹ methoxyamine hydrochloride in pyridine. The sample was then treated with 40 μ l 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 \times 0.25 mm, 0.25 μ m film thickness). Samples (1 μ l) 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 NaHCO₃ 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 NaHCO₃ (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 alkalization 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

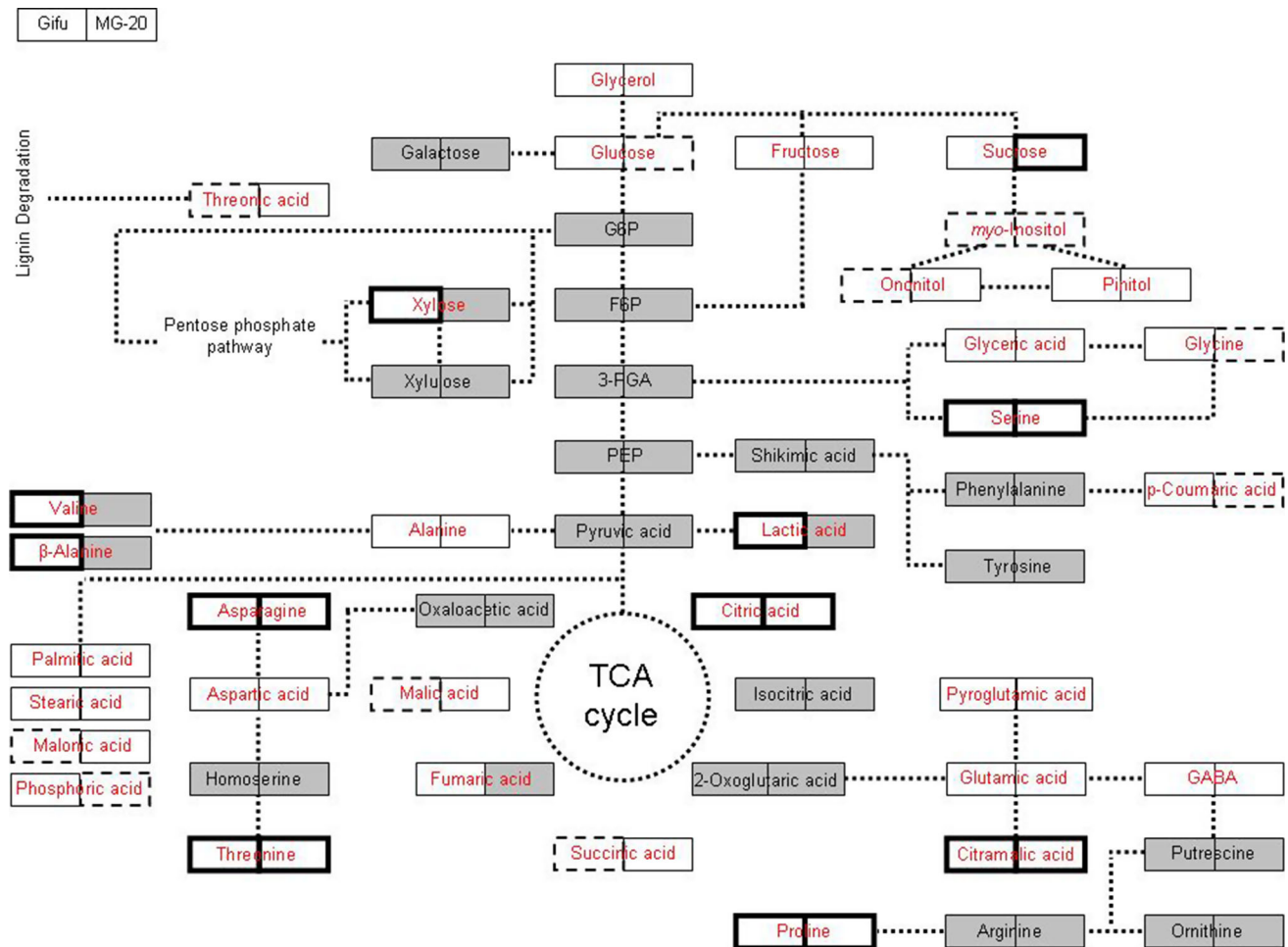


Fig. 1 Scheme of significant changes in metabolites abundance after alkaline treatment in Gifu B-129 (right rectangles) and MG-20 (left rectangles) shoots. Arrows indicate one or more steps pathways between two metabolites. Dashed rectangle frames indicate significantly decreased and thickened frames 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; Roschztardt et al. 2011; Rellán-Álvarez et al. 2010; Roschztardt 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 (Babu et al. 2014). This result, along with the alkaline induction of a zinc metalloprotease 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 alkalized 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 alkalized 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 alkalized ecotypes, and a not yet identified metabolite (here referred as ui014) that were detected exclusively on alkalized 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 alkalized 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 alkalized 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 alkalized 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 alkalization.

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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References

- Babuín MF, Campestre MP, Rocco R et al (2014) Response to long-term NaHCO₃-derived alkalinity in model *Lotus japonicus* Ecotypes Gifu B-129 and Miyakojima MG-20: transcriptomic profiling and physiological characterization. *PLoS One* 9:e97106. doi:10.1371/journal.pone.0097106
- Barnett NM, Naylor AW (1966) Amino acid and protein metabolism in Bermuda grass during water stress. *Plant Physiol* 41:1222–1230
- Bie Z, Ito T, Shinohara Y (2004) Effects of sodium sulfate and sodium bicarbonate on the growth, gas exchange and mineral composition of lettuce. *Sci Hortic (Amsterdam)* 99:215–224. doi:10.1016/S0304-4238(03)00106-7
- Colebatch G, Desbrosses G, Ott T et al (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant J* 39:487–512. doi:10.1111/j.1365-313X.2004.02150.x

- Desbrosses GG, Kopka J, Udvardi MK (2005) *Lotus japonicus* metabolic profiling. Development of gas chromatography–mass spectrometry resources for the study of plant-microbe interactions. *Plant Physiol* 137:1302. doi:[10.1104/pp.104.054957.1302](https://doi.org/10.1104/pp.104.054957.1302)
- Di Rienzo JA, Casanoves F, Balzarini MG et al (2010) InfoStat versión 2010
- Díaz P, Borsani O, Monza J (2005) *Lotus*-related species and their agronomic importance. In: Marquez AJ (ed) *Lotus* Japan handbook. Springer, The Netherlands, pp 25–37
- Ding YZ, Song ZG, Feng RW et al (2014) Interaction of organic acids and pH on multi-heavy metal extraction from alkaline and acid mine soils. *Int J Environ Sci Technol* 11:33–42
- Duda CT, Cherry JH (1971) Chromatin- and nuclei-directed ribonucleic acid synthesis in sugar beet root. *Plant Physiol* 47:262–268
- Escaray FJ, Menéndez AB, Gárriz A et al (2012) Ecological and agronomic importance of the plant genus *Lotus*. Its application in grassland sustainability and the amelioration of constrained and contaminated soils. *Plant Sci* 182:121–133. doi:[10.1016/j.plantsci.2011.03.016](https://doi.org/10.1016/j.plantsci.2011.03.016)
- Fiehn O, Kopka J, Dörmann P et al (2000) Metabolite profiling for plant functional genomics. *Nat Biotechnol* 18:1157–1161
- Gilbert GA, Gadush MV, Wilson C, Madore MA (1998) Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth during salinity stress. *J Exp Bot* 49:107–114. doi:[10.1093/jxb/49.318.107](https://doi.org/10.1093/jxb/49.318.107)
- Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. *Annu Rev Plant Physiol* 31:149–190
- Guo R, Yang Z, Li F et al (2015) Comparative metabolic responses and adaptive strategies of wheat (*Triticum aestivum*) to salt and alkali stress. *BMC Plant Biol* 15:170–183
- Hageman RH (1984) Ammonium versus nitrate nutrition of higher plants. In: Hauck RD (ed) Nitrogen in crop production. ASA, CSSA, SSSA, Madison, pp 67–85
- Hatfield RD, Marita JM (2010) Enzymatic processes involved in the incorporation of hydroxycinnamates into grass cell walls. *Phytochem Rev* 9:35–45
- Heldt H-W, Piechulla B (2011) Plant biochemistry, 4th edn. p 656
- Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. *Calif Agric Exp Stn Circ* 347:1–32
- Hu L, Zhang P, Jiang Y, Fu J (2015) Metabolomic analysis revealed differential adaptation to salinity and alkalinity stress in kentucky bluegrass (*Poa pratensis*). *Plant Mol Biol Rep* 33:56–68
- Iwata Y, Koizumi N (2012) Plant transducers of the endoplasmic reticulum unfolded protein response. *Trends Plant Sci* 17:720–727. doi:[10.1016/j.tplants.2012.06.014](https://doi.org/10.1016/j.tplants.2012.06.014)
- Kopka J, Schauer N, Krueger S et al (2005) GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics* 21:1635–1638
- Kumar K, Kumar M, Kim S-R et al (2013) Insights into genomics of salt stress response in rice. *Rice* 6:27. doi:[10.1186/1939-8433-6-27](https://doi.org/10.1186/1939-8433-6-27)
- Lisec J, Schauer N, Kopka J et al (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat Protoc* 1:387–396
- Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. p 651
- Nikiforova V, Freitag J, Kempa S et al (2003) Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. *Plant J* 33:633–650
- Pang Q, Zhang A, Zang W et al (2016) Integrated proteomics and metabolomics for dissecting the mechanism of global responses to salt and alkali stress in *Suaeda corniculata*. *Plant Soil*. doi:[10.1007/s11104-015-2774-0](https://doi.org/10.1007/s11104-015-2774-0)
- Paz RC, Rocco RA, Reinoso H et al (2012) Comparative study of alkaline, saline, and mixed saline-alkaline stresses with regard to their effects on growth, nutrient accumulation, and root morphology of *Lotus tenuis*. *J Plant Growth Regul* 31:448–459. doi:[10.1007/s00344-011-9254-4](https://doi.org/10.1007/s00344-011-9254-4)
- Rao KP, Rains D-W (1976) Nitrate absorption by Barley: I. Kinet Energ Plant Physiol 57:55–58. doi:[10.1104/pp.57.1.55](https://doi.org/10.1104/pp.57.1.55)
- Rellán-Álvarez R, Giner-Martínez-Sierra J, Orduna J et al (2010) Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. *Plant Cell Physiol* 51:91–102
- Roessner U, Luedemann A, Brust D et al (2001) Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. *Plant Cell* 13:11–29
- Roschztardt H, Grillet L, Isaure MP et al (2011) Plant cell nucleolus as a hot spot for iron. *J Biol Chem* 286:27863–27866. doi:[10.1074/jbc.C111.269720](https://doi.org/10.1074/jbc.C111.269720)
- Roschztardt H, Conéjéro G, Divol F et al (2013) New insights into Fe localization in plant tissues *Front. Plant Sci* 4:350. doi:[10.3389/fpls.2013.00350](https://doi.org/10.3389/fpls.2013.00350)
- Rose MT, Rose TJ, Pariasca-Tanaka J et al (2012) Root metabolic response of rice (*Oryza sativa* L.) genotypes with contrasting tolerance to zinc deficiency and bicarbonate excess. *Planta* 236:959–973
- Schauer N, Steinhauser D, Strelkov S et al (2005) GC–MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Lett* 579:1332–1337
- Shahidi F, Chandrasekara A (2010) Hydroxycinnamates and their in vitro and in vivo antioxidant activities. *Phytochem Rev* 9:147–170
- Sheveleva E, Chmara W, Bohnert HJ, Jensen RG (1997) Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiol* 115:1211–1219. doi:[10.1104/pp.115.3.1211](https://doi.org/10.1104/pp.115.3.1211)
- Szabados L, Kovacs H, Zilberstein A, Bouchereau A (2011) Plants in extreme environments: importance of protective compounds in stress tolerance. *Adv Bot Res* 57:105–150
- Taji T, Takahashi S, Shinozaki K (2006) Inositols and their metabolites in abiotic and biotic stress responses. In: Majumder AL, Biswas BB (eds) *Biology of inositols and phosphoinositides SE-10*. Springer, New York, pp 239–264
- Valdez-Aguilar LA, Reed DW (2010) Growth and nutrition of young bean plants under high alkalinity as affected by mixtures of ammonium, potassium, and sodium. *J Plant Nutr* 33:1472–1488. doi:[10.1080/01904167.2010.489985](https://doi.org/10.1080/01904167.2010.489985)