ITALO ANTUNES PEREIRA LIMA

ON THE ROLE OF CENTRAL METABOLISM FOR THE DIFFERENTIAL ALUMINUM TOLERANCE IN THREE *Arabidopsis thaliana* ECOTYPES

Dissertation presented to the Universidade Federal de Viçosa as part of requirement of the Plant Physiology Graduate Program for the obtention of the degree of *Magister Scientiae.*

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APPROVED: February 27th, 2019

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To José Eustácio Bahia Lima e Marinalva Silva Pereira Lima,
my dear parents, I dedicate.

Epigraph

“To see what we have before us requires a constant struggle.”

Eric Arthur Blair (George Orwell)

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ABSTRACT

LIMA, Italo Antunes Pereira, M.Sc., Universidade Federal de Viçosa, February, 2019. **On the role of central metabolism for the differential aluminum tolerance in three *Arabidopsis thaliana* ecotypes.** Adviser: Wagner Luiz Araújo. Co-adviser: David Barbosa Medeiros.

Aluminum (Al) toxicity is for long known to promote severe impairments to the production of many crops in acid soils. In order to overcome toxicity, Al resistance genes have been characterized in different plant species and are involved in two main mechanisms: (*i*) tolerance, regarding the Al detoxification within the plant cell and (*ii*) exclusion, which involves Al complexation in the rhizosphere by releasing of organic acids (OAs). Plants using the Al-induced OA exudation as the main mechanism might spend valuable carbon commodities during this process. Therefore, the OA exudation must be tightly regulated and closely related to the carbon metabolism. In this study, growth and yield parameters were used to compare the response of three Arabidopsis ecotypes namely Columbia-0 (Col-0), Wassilewkija (Ws), and Landsberg erecta (Ler), under Al stress, which was correlated with expression of Al-responsive genes. Further, a detailed metabolic characterization was also performed in 28-day-old plants grown in hydroponic system. The so-called Al-sensitive ecotypes, Col-0 and Ws, presenting higher yield and growth under control conditions, exhibited stronger reductions in these parameters under long-term Al exposure. Ler, classified as the most capable in dealing with Al stress, showed relative smaller reductions comparing control and Al-stress conditions. All the ecotypes presented high expression of the well-known Al-resistance gene *AtALMT1.* Col-0 and Ws plants under Al stress increased leaf fructose and starch contents, suggesting a low carbon use efficiency (CUE). Similarly, higher increases in amino acids, carbohydrates and organic acids levels occurred in both shoot and roots tissues of Col-0 and Ws plants, but not of Ler. Altogether, our results suggest that higher capacity of using and translocate reduced carbon molecules seems to be crucial to Arabidopsis overcome Al stress. In addition, lower expenses in carbon molecules might be linked to a higher capacity to deal with Al stress in long-term experiments. Overall, our results support a tight relationship between primary metabolism and Al stress responses. The results obtained are discussed within the context of current models of Al resistance mechanism providing novel insights on the influence of Al stress on plant growth and primary metabolism in leaves and shoots.

RESUMO

LIMA, Italo Antunes Pereira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **O papel do metabolismo central na tolerância diferencial ao alumínio em três ecótipos de *Arabidopsis thaliana*.** Orientador: Wagner Luiz Araújo. Coorientador: David Barbosa Medeiros.

A toxicidade do alumínio (Al) é há muito tempo conhecida por promover graves prejuízos à produção de muitas culturas em solos ácidos. Para superar essa toxicidade, genes de resistência ao Al foram caracterizados em diferentes espécies de plantas e estão envolvidos em dois mecanismos principais: (i) tolerância, referente à desintoxicação do Al dentro da célula vegetal e (ii) exclusão, que envolve a complexação do Al na rizosfera pela liberação de ácidos orgânicos (AOs). As plantas que utilizam a exsudação de AO induzida por Al como principal mecanismo, dispendem de *commodities* de carbono valiosas neste processo. Portanto, a exsudação AO deve ser finamente regulada e intimamente relacionada ao metabolismo do carbono. Neste estudo, os parâmetros de crescimento e produtividade foram utilizados para comparar a resposta de três ecótipos de Arabidopsis, Columbia-0 (Col-0), Wassilewkija (Ws) e Landsberg erecta (Ler), sob estresse por Al, que foi correlacionada com expressão de genes responsivos a este estresse. Além disso, uma caracterização metabólica detalhada foi realizada em plantas com 28 dias de idade, cultivadas em sistema hidropônico. Os ecótipos classificados como sensíveis ao Al, Col-0 e Ws, cuja produção e crescimento sob condições controle são maiores em ralação ao Ler, exibiram reduções mais drásticas desses parâmetros sob exposição ao Al no longo prazo. Ler, classificado como o mais capaz em lidar com o estresse por Al, exibiu reduções relativamente menores comparando as condições de controle e estresse. Todos os ecótipos apresentaram alta expressão do principal gene de tolerância ao Al em Arabidopsis, *AtALMT1*. As plantas Col-0 e Ws sob estresse por Al aumentaram o teor de frutose e amido em folhas, sugerindo uma baixa eficiência de uso de carbono (EUC). Da mesma forma, maiores aumentos nos níveis de aminoácidos, carboidratos e ácidos orgânicos ocorreram em ambos os tecidos de folhas e raízes plantassem Col-0 e Ws, mas não em Ler. Em conjunto, os resultados sugerem que a maior capacidade de usar e translocar moléculas de carbono reduzidas parecem ser cruciais para Arabidopsis superar o estresse por Al. Ademais, menores gastos em moléculas de carbono em processo de exsudação podem estar ligados a uma maior capacidade de lidar com o

estresse em experimentos de longo prazo. No geral, nossos resultados suportam uma estreita relação entre o metabolismo primário e as respostas ao estresse por Al. Os resultados obtidos são discutidos dentro do contexto dos atuais modelos de mecanismo de resistência ao Al, fornecendo novos insights sobre a influência deste estresse sobre o crescimento das plantas e metabolismo primário em folhas e raízes
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1. INTRODUCTION

Acid soils, where aluminum (Al) toxicity is considered a major factor reducing crop yield , are globally widespread and are prevalent in developing countries (Kochian et al., 2015). Approximately 50% of the world's arable land is classified as acidic with soil pH below 5 (Uexkull and Mutert, 1995). As well known, low pH facilitates the Al solubilization as the rhizotoxic cation Al3+, damaging and inhibiting root growth, consequently reducing both water and nutrient uptake, and therefore affecting directly crop yield (Kochian et al., 2015). Soil acidity per se is a limiting factor caused by proton (H^+) and manganese (Mn^{2+}) toxicity coupled with nutritional deficiency of important essentials macronutrients such as phosphorus (P), calcium (Ca^{2+}) and magnesium (Mg^{2+}) (Iuchi et al., 2007). Moreover, under low soil pH (below 5.0) Al can be solubilized into different ionic forms (e.g. $Al(OH)_2^+$, $Al(OH)^{2+}$ and Al^{3+}), which are considerably toxic to plants. Notably, the trivalent ion (Al^{3+}) is assumedly the most toxic to plants, and has the greater ability to interfere in root growth and development even at micromoles concentrations (Kochian et al., 2004). Although roots are recognized as the most sensitive organ in relation to Al stress, aerial parts are also known to be widely affected by this metal (Li and Xing, 2011; Mattiello et al., 2014).

The excess of Al in low pH soils facilitates the contact of this cation with the roots resulting in growth and function inhibitions through physical and cellular mechanisms, ultimately reducing water absorption and nutrient acquisition (Gupta et al., 2013). According to Illéš et al. (2006) the potential Al toxicity may result from complex interactions with the cell wall (apoplast), plasma membrane and/or cytosol (simplast) promoting the reactive oxygen species (ROS) generation (Kochian et al., 2004). Al can also directly alter, in an irreversible manner, the DNA physical structure, affecting, secondarily, the cell cycle of plants and stopping root growth through changes in differentiation process of stem cells (Eekhout et al., 2017; Horvath et al., 2017). In fact, the root distal transition zone (DTZ) is considered the root region most susceptible to Al (Kopittke et al., 2015).

Throughout plant evolution, the development of different resistance mechanisms occurred in order to mitigate the damages caused by Al. Conceptually, these mechanisms have been well-classified as tolerance and exclusion mechanisms. Briefly, the tolerance mechanism is characterized by the sequestration

with internal detoxification of Al, whereas the exclusion mechanism prevents the Al ion to reach the root apex by the exudation of organic acids (OA) by a complex and large transport proteins family (Delhaize et al., 2007; Kochian et al., 2015). The resistance mechanisms vary among species and involve specific transporters. Therefore, differential detoxification of phytotoxic Al species using OA, such as malate, citrate, and oxalate, has been reported in several plant species, promoting cation chelation and ultimately preventing the Al contact to the root plasma membrane (Kochian et al., 2004). In addition, other compounds have also been related to Al resistance such as catechol, catechin, and quercetin (Kidd et al., 2001; Kochian et al., 2015). The principal OA used to chelate Al in the rhizosphere of both wheat (*Triticum aestivum*) and Arabidopsis (*Arabidopsis thaliana*) is malate and it involves the Al-activated malate transporter *Ta*ALMT1 and *At*ALMT1 ion channels, respectively (Sasaki et al., 2004; Kobayashi et al., 2007). In addition, the family of antiport transporters, AO/H^+, multidrug and toxic compound extrusion (MATE), acts on citrate transport using specifically *Sb*MATE1 in sorghum (*Sorghum bicolor*) (Magalhaes et al., 2007) and *Zm*MATE1 in maize (*Zea mays*) (Maron et al., 2010). It should be noted that, for certain species, both carrier families can act to confer tolerance independently as occurs in Arabidopsis. In fact, the *At*ALMT1 and AtMATE1 genes were characterized as functional in response to Al stress, although AtALMT1 gene is seemingly more important for this species than the AtMATE1 (Liu et al., 2009). Nevertheless, the exudation of OA as well as the usage of phenolic compounds represent expenditures of reduced carbon compounds to overcome the negative impacts of Al stress in the rhizosphere or within root cells (Kochian et al., 2015) .

In species such as rice (*Oryza sativa*), recognized as one of the most tolerant to Al, buckwheat (*Fagopyrum esculentum*) and hydrangea (*Hydrangea macrophylla*), the exudation of OA (exclusion mechanism) is apparently not the main mechanism (Kochian et al., 2015). Accordingly, other genes have been characterized as the main promoters of Al resistance dealing with the toxic ion within the plant cells (Kochian et al., 2015). In rice, in addition to the exudation of OA, particularly citrate mediated by the MATE gene (*OsFRDL4*), changes in cell wall composition (Ying et al., 2013; Zhu et al., 2014) and internalization of the apoplastic Al followed by Al sequestration into vacuoles organelle have been reported (Yokosho et al., 2011). The ability to

internalize Al ions, lowering its toxic level in the cell wall, is due to specific ion transporter family present in the plasma membrane, such as the Nramp aluminum transporter 1 (*Os*Nrat1) in rice (Xia et al., 2010), and the *Hydrangea macrophylla*, plasma membrane aluminum transporter 1 (*Hm*PALT1) (Negishi et al., 2012). Once inside the cells Al can be either complexed by OA and/or phenolic compounds or transported into the vacuoles through tonoplast transporters following by complexation (Kochian et al., 2015). The vacuolar ABC transporter (*Os*ALS1) in rice (Huang et al., 2009) and the vacuolar aluminum transporter 1 (HmVALT) in *Hydrangea macrophylla* (Negishi et al., 2012) have been characterized as Al transporters at the tonoplast and their orthologues were assumed of paramount importance for the resistance mechanism in some plant species (Kochian et al., 2015).

The resistance mechanisms are both closely related to the mitochondrial metabolism (Nunes-Nesi et al., 2013). Besides of being the main source of OA, the tricarboxylic acid (TCA) cycle plays an essential role in ATP biosynthesis in the mitochondria through oxidative phosphorylation in heterotrophic tissues and synthesis of reducing equivalents (*i.e*. NADH, FADH2 and NAD(P)H) (Fernie et al., 2004; Nunes-Nesi et al., 2013). Briefly, the TCA cycle can operate in two distinct ways, a cyclic mode responsible for the synthesis of ATP and reducing equivalents, and in a non-cyclic manner depending on the metabolic and physiological demands of the cell (Sweetlove et al., 2010), for example, under stress conditions. According to Igamberdiev and Eprintsev (2016), the OA represent transitory or fixed carbon storage forms and are important intermediates of the TCA cycle. In addition, these OA participate, to a lesser extent, in the glyoxylate cycle in plants (Cavalcanti et al., 2014; Sharma et al., 2016). Thus, OA including TCA cycle intermediates, have crucial importance for the most diverse biochemical processes as energy production, biosynthetic pathways, maintenance of redox and ionic balance, making these molecules key elements in plant adaptations to different environments (Igamberdiev and Eprintsev, 2016).

In response of Al metabolic deviations and incomplete operation of the TCA cycle as well as changes in ATP synthesis in *Pseudomonas fluorescens* were observed (Singh et al., 2009; Lemire et al., 2010). This fact apart, the precise mechanism by which Al affect processes such as plant photosynthesis and

mitochondrial metabolism are still poorly understood (Nunes-Nesi et al., 2013). However, is already known that Al stress might decrease photosynthesis rate associated with changes in the chlorophyll content in rice, rye (*Secale cereale*), and *Artemisia annua L.* (Aftab et al., 2010; Silva et al., 2012; Yang et al., 2013; Junior et al., 2014). Noteworthy, the role of TCA cycle in response to Al is still elusive, with indications that metabolism is linked to Al stress in plants, such as in Arabidopsis, where energy metabolism genes were triggered as well as in maize, in which was found an Al-induced upregulation of genes encoding TCA cycle enzymes (Kumari et al., 2008; Mattiello et al., 2014).

Into the metabolic alteration context, it is important to mention that the non-cyclic flow of the TCA cycle allows the deviation of malate and citrate to other routes. Thus, it is reasonable to assume that alterations in the levels of these OA by their exudation under Al stress can impact directly central metabolism, therefore requiring a fine energetic and metabolic regulation. The use of OA as a mechanism of resistance in plants leading into a significant carbon cost shows that this exudation process must be tightly regulated, both spatially (in root apices) and in response to the presence of Al (Kochian et al., 2015). In this vein, the presence of Al promotes fast responses such as increases in the expression of *ALMT* and *MATE* genes, which are reduced when the stress is withdrawn (Kobayashi et al., 2007; Magalhaes et al., 2007). Al also increases OA exudation via post-translational regulation of the above mentioned transporters (Pineros et al., 2008). Noteworthy, those genotypes whose constitutive or basal expression of the resistance genes is naturally higher present higher resistance to Al stress given that the patterns of expression are correlated with the physiological capacity of AO exudation (Sasaki et al., 2004; Ezaki et al., 2013; Kochian et al., 2015).

The basal gene expression or the elevation in response to the stress are usually controlled by transcription factors. In Arabidopsis, the *AtALMT1* gene appears to be regulated by three transcription factors namely *AtSTOP1*, *AtSTOP2* and *AtWRKY46* (Sawaki et al., 2009; Ding et al., 2013; Kobayashi et al., 2014). The *STOP* (Sensitive to Proton Rhizotoxicity) genes are zinc-finger type transcription factors that are related to the response to Al and low pH stresses and positively regulates the expression of *ALMT1* and *MATE1* genes. In contrast, WRKY46 belongs to a group of proteins containing the WRKY domain with a particular motif pattern

(Eulgem et al., 2000) which negatively regulates only the expression of *ALMT1* gene. Knockout mutants for *WRKY46* showed increased and faster expression of *AtALMT1* in the first hours of stress exposure, which was correlated with increased malate exudation and, consequently, higher resistance to Al (Ding et al., 2013). The presence and activity of transcription factors regulating the exudation of OA is important to avoid the indiscriminate loss of important respiratory and metabolic commodities such as malate and citrate (Kochian et al., 2015). Recently, Liu et al. (2012) demonstrated that the *AtALMT1* and *AtMATE* genes are expressed in different regions of the Arabidopsis roots, namely root apices and mature portions of the roots, respectively. It was found that expressing *AtMATE* gene under *AtALMT1* promoter increased citrate exudation and, due to its higher Al detoxification capacity, also elevated the carbon use efficiency (CUE) following Al stress conditions (Liu et al., 2012). Nevertheless, little is currently known about the real impact of Al resistance mechanisms in deviating respiratory substrates, as well as the negative metabolic effects caused by Al in autotrophic (leaves) and heterotrophic (roots) organs. Taken together, all this information demonstrates the importance of genetic engineering in the development of more efficient materials in the use of organic molecules to deal with Al toxicity, and it might be possible to develop more resistant and yet productive materials with lower yield penalties in crops.

The previous study from Toda et al. (1999) attempted to better understand the differential Al stress responses by using three Arabidopsis ecotypes; Columbia-0 (Col-0), Wassilewkija (Ws), and Landsberg erecta (Ler), originally from United States, Russia, and Germany, respectively. Remarkably, the natural genetic variation of the three Arabidopsis ecotypes led to differential tolerance only measured by the root growth under hydroponic conditions and the rosette diameter in plants grown in soil with different Al availability. Briefly, it was shown that the responses obtained under hydroponics and soil conditions correlated to interpret tolerance responses to Al. Additionally, it was also demonstrated that among the three ecotypes, Ws was considered the most tolerant, followed by Col-0, whereas Ler was the most sensitive to Al stress under the conditions studied. This study enhanced our knowledge on the differential tolerance to Al in Arabidopsis, but the metabolic impacts underlying Al responses have not been examined yet. Thus, in addition to quantitative growth measurements, here we evaluated in detail the responses of central metabolism and

expression of key genes associated with Al resistance in order to correlate the putative metabolic impacts with the previous reported differential tolerance in the studied Arabidopsis ecotypes.

Our results provide novel physiological insights into how and to which extent three different Arabidopsis ecotypes react to toxic Al in both leaves and roots tissues following both short and long period of Al exposure. Biometric analyses coupled with detailed metabolic profiling demonstrated ecotype and tissue-specific response patterns. From a metabolic point of view, shoot and roots showed previously unnoticed metabolic shifts in response to Al stress. In summary, the work presented here aid in the elucidation of the metabolic responses following Al stress allowing us to refine our understanding of complex response of plants to this environmental constraint.

2. MATERIAL AND METHODS

2.1. Plant growth conditions and aluminum treatment

Three ecotypes of *Arabidopsis thaliana* namely Columbia (Col-0), Wassilewskija (Ws) and Landsberg (Ler) were used. Geographical origin of the accessions is additionally available at VNAT (http://dbsgap.versailles.inra.fr/vnat/). New seeds from the three ecotypes were obtained from plants cultivated in soil under the same growth condition, such as short-day (8 h/16 h of light/dark), irradiance of 150 µmol m^{-2} s^{-1}, 22ºC/20ºC during the light/dark cycle, and 70 ± 5% relative humidity. Seeds were surface-sterilized by incubation in 1 mL of 70% (v/v) ethanol for 1 min, followed by manual shaking for 2 min. After that, the ethanol was replaced by 2.5% (v/v) sodium hypochlorite and shacked manually for 2 min and kept on room temperature 15 min. Subsequently, the seeds washed five times by adding 1 ml sterilized water, inverting the tubes for 2 min and spinning the seeds down. After the sterilization procedure, the seeds passed through the vernalization process at 4.0º C for 48h.

Plant cultivation took place in a specialized hydroponic system as previously described by Monte-bello et al. (2018). Seeds were sown over pipette tip hacks covers filled with sterile half strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) nutrient solution and 0.5% (*w/v*) phytagel (Sigma-

Aldrich), and the reservoir (pipette box) filled with a hydroponic solution as detailed in Zhu et al. (2012) including 0.05% (w/v) MES buffer and pH 5.7 adjusted with 1M KOH (Fig. 1). Plants were cultivated in a growth chamber under short-day conditions (8h/16h of light/dark), irradiance of 150 µmol photons.m^{-2}. s^{-1}, temperature of 20 ± 2° C and humidity of 70± 5%. During all the experiment set-up, the nutritive solution, as described above, was regularly monitored and changed as necessary aiming the pH range maintenance, solution temperature and completely sterile. 25 days after sowing, homogenous plants were selected and transferred to a new hydroponic nutrient solution with mild low pH (5.0) where the selected plants remained three days under acclimation conditions for the acclimatization process. Finally, 28 days-old plants were divided into two different groups, namely under 50µM $AlCl_3$ (treatment, $AlCl_3$ Anhydrous stock solution - Sigma-Aldrich) and 0 µM $AlCl_3$ (control), both under pH 4.5. Al-stress treatment started 1h after the beginning of the light period and all plants were maintained at the same conditions for the next 120h. Plant material (leaves and roots) was harvested at the following time points (Fig. 1): at the beginning of the experiment (0h after treatment, only control plants), 24h, 72h and 120h after treatment for both, control and Al^{3+}, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. For each time point and condition three individual Arabidopsis plant were harvested, generating a composite sample for each repetition, in a total of five repetitions. Three independent experiments were performed to collect plant material for metabolism and gene expression, root respiration and histochemical tests for ROS identification. Additionally, the experiment was repeated at least three times (even in different growth facilities) with similar phenotypes observed each time.

2.2. Evaluation of germination and root growth rate

The impacts of Al over seed germination and root growth rates were analyzed in Petri dishes (agar plates) with nutrient solution as previously described (Zhu et al., 2012). Briefly, to evaluate germination, and root growth four different treatments were used, namely pH 5.7 (general control), pH 4.5 (control treatment without Al) and two Al concentrations (25 and 100 µM $AlCl_3$) predetermined by screening test. To ensure Al^{3+} availability in the solution, both treatments and controls had the pH adjusted to 4.5. Due to a low Agar's polymerization efficiency under low pH, we used 1% (w/v)

instead of 0.7% of agar as in the control (pH 5.7). The seeds sterilization procedure and experimental condition were as described before. Following exposition to the light, the evaluation of germination kinetic occurred at 24h, 30h, 36h, 48h, 60h and 72h, and for root growth rate, seeds were previously placed 3 days in the light to seed germination, then the measurements occurred every 2 days during 12 days after germination.

2.3. Histochemical ROS assays

Qualitative evaluation of (ROS) in leaves and roots was performed by histochemical test for peroxide (H_2O_2) and superoxide(O_2^-) as previously described (Kong et al. (2011) with modifications in time exposure and reagents concentration. Once young Arabidopsis tissues are tender, we used lower exposure time to avoid dyes over staining. Thus, the exposure time was 30 and 15 min for leaves and roots, respectively. For the identification of H_2O_2 species, it was used the 3,3'-Diaminobenzidine (DAB), 1.0 $mg.ml^{-1}$ and for the O^{2-}. species it was used Nitrobluetetrazolium (NBT) 0.1 mg mL^{-1}, as previously described by Kong et al. (2011). After the exposure time with the aforementioned reagents, the removal of pigments was necessary for leaves, so the staining solution (*i.e.* DAB or NBT) was removed and a distaining solution (ethanol: acetic acid: glycerol 3:1:1) was added to 50 mL tube until all the samples were completely covered and further cooked in a water bath for 15 minutes at 90 ± 5°C. Next, the distaining solution was removed, and a new distaining solution was added allowing storage until samples were photographed. Photos were analyzed using ImageJ software. The pictures were previously converted to 8-bit gray scale archive, the tissue area was delimited, and the stained area was identified by the program generating the percentage (%) of affected (stained) area.

2.4. Metabolite analyses and biochemical assays

Samples from the hydroponics experiment were harvested in the middle of the light period and were flash frozen in liquid nitrogen and stored at -80 ºC until further analyses. The photosynthetic pigments were determined in leaves before chloroform addition, as in Porra et al. (1989). The levels of malate and fumarate were measured as described in Nunes-Nesi et al. (2007), whereas the quantification of starch,

glucose, fructose and sucrose (Fernie et al., 2001), free amino acids (Sienkiewicz-porzucek et al., 2010) and total proteins (Bradford, 1976). The metabolite profiling was carried out in samples harvested at the middle of the light period for both leaves and roots via gas chromatography coupled to mass spectrometry (GC-MS) exactly as previously described by Lisec et al. (2006). Briefly, the extraction was performed using 700 µL of methanol and shaking (800 rpm) at 70 ºC for 15 minutes, 60 µL of Ribitol (0.2 mg mL^{-1}) was added as an internal standard. After heating, the samples were centrifuged, and supernatant was separated from pellet. To the supernatant was add 375 µL chloroform (for HPLC >99.9%) and 750 µL mili-Q water. Samples were centrifuged, and the aqueous phase was separated for future analysis. Chromatograms were manually evaluated using the TagFinder software (Luedemann et al., 2011), using the reference library available in the Golm Metabolome Database (Kopka et al., 2005) and following the recommended reporting format (Fernie et al., 2011).

2.5. Root respiration rate

In order to evaluate the respiration rate of roots in response to Al stress the O_2 consumption was measured using Clark-type electrode (www.hansatech-instruments.com) and OxyTrace$^+$ software and the measurements were made in a liquid-phase respiration according to the manufacturer's instructions. The electrode assembly was done according to González et al. (2001). The liquid-phase assessments were carried out using air saturated distilled water (mili-Q water - Millipore Corporation), with the chamber temperature set to 25°C. For the electrode disc preparation, the cathode was moistened with a drop of KCl (5% v/v) and covered with Teflon membrane avoiding wrinkle formations. The calibration was carried out with 2 mL of air saturated water and the "zero" oxygen concentration was obtained by adding sodium dithionite in the reaction vessel and waiting for the stable plateau. After calibration, the chamber was cleaned with distilled water before measurements. Each measurement was carried out by evaluating the respiration rate of at least two whole roots with 1.5 mL of mili-Q water inside the chamber. Afterwards, roots samples were oven dried at 60°C for 48 hours and weighted in order to acquire the dry weight (DW) for further data normalization. The measurement time of the

samples was approximately 10 minutes, and the data of conductivity to μmol O_2 min^{-1}.g DW^{-1} consumed (respired) was transformed using the following equation:

$$\mu mol\ O_2 min^{-1}\ .\ \mathrm{g}\ DW^{-1} = \frac{WOC\ .Vol\ .(fcond - icond)}{wc\ .\ t^{-1}\ .DW}$$

Where:

WOC: Oxygen content of air saturated water at 30°C (0.230 μmol. mL^{-1});

Vol: Water volume in the reaction vessel;

fcond: final reading of conductivity;

icond: initial reading of conductivity;

Wc: water conductivity;

t: minutes between readings;

DW: sample dry weight.

2.6. Gene expression

Quantitative real-time PCR (qRT-PCR) analysis was performed with total RNA isolated from leaves and whole roots separately. RNA extraction was performed using TRizol® reagent (Ambion, Life Technology) following the manufacturer's manual. The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a Nanodrop ND-1000 spectrophotometer (http://www.nanodrop.com/). Digestion with DNase I (Ambion; http://www.ambion.com/) was performed according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen; http://www.invitrogen.com/) according to the manufacturer's instructions. The efficiency of cDNA synthesis was estimated by semi-quantitative PCR using two primer pairs amplifying 5' and 3' regions of the constitutive gene Actin (forward [5'- CTTGCACCAAGCA GCATGAA -3'] and reverse [5'- CCGATCCAGACACTGTACTTCCTT-3']. For analysis of gene expression, the Fast SYBR® Green PCR Master Mix was used with the MicroAmpTM Optical 96-well Reaction Plate and MicroAmpTM Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA). The relative expression levels were normalized using the constitutively expressed gene ACTIN and calculated using the 2-ΔΔCT method as discribed by Livak and Schmittgen (2001).The primers used for qRT-PCR were designed using the QuantiPrime software (http:www.quantprime.de) (Arvidsson et al., 2008). Detailed primers information is additionally described in the Supplemental

Table S1. The following genes were analysed: *ALUMIUM ACTIVATED MALATE TRASNPORTER 1* (*ALMT* 1), *WRKY 46*, *SENSITIVE TO PROTON RHYZOTOXICITY 1* (*STOP 1*), *MULTIDRUG AND TOXIC COMPOUND EXTRUISION 1* (*MATE 1*), *SUCROSE-PROTON SYMPORTER 2* (*SUC 2*), *GLUTATHIONE S-TRANSFERASE 1* (*GST1*) and *ZAT 12*.

2.7. Evaluation of biometric parameters of seeds and final productivity

To further investigate the phenotype in response of Al treatment, plants of each ecotype that were submitted to the 5 days stress under low pH (4.5) and low pH with 50 µM Al were kept under hydroponic conditions until their reproductive phase, maintaining the pH control as 4.5 and enhancing the Al concentration in the treatment to 100 µM Al with pH 4.5 as well. Nutrient solution was changed every 3-4 days since there was no aeration. During the reproductive phase and at the end of their life cycle, siliques were collected for biometric analyses and the number of siliques was determined. Harvested siliques were cleared with 0.2 M NaOH and 1% Sodium Dodecyl Sulfate (SDS) solution to remove pigments. Images of the siliques were taken with a digital camera (Canon Powershot A650 IS) attached to a stereomicroscope (Zeiss Stemi 2000-C). The measurement of silique length was performed on the images using ImageJ software. Weighing aliquots of seeds and counting the number of each batch determined seed weight. Total seed yield was determined by using the number of siliques multiplied by the number of seeds per silique of each plant.

2.8. Experimental design and statistical analysis

The data were obtained from experiments conducted in a completely randomized design. Additionally, the complete setup was independently repeated at least three times with similar phenotypes observed each time. Data was expressed as means ± standard error (SE). All data passed the normality and equal variance Kolmogorov-Smirnov tests and then the means were further analyzed by a one-way analysis of variance and some cases in two-way, when necessary. In the absence of restrictions, the data was submitted to analysis of variance (P<0.05) and comparison of means by the Tukey test (P<0.05) using the GENES software (Cruz, 2013).

3. RESULTS

3.1. Differential aluminum tolerance in Arabidopsis ecotypes under hydroponic culture and agar plate systems revealed by growth parameters

In order to understand the effects caused by the Al stress in Arabidopsis plants, we adapted a hydroponic system previously developed by Monte-Bello et al. (2018), (Fig. 1). Plants of 28 days old previously grown under normal conditions were selected and submitted to the experiment that consisted of two treatments, being pH 4.5 as control and pH 4.5 added with 50 µM Al as the stress treatment. Both shoot and roots were harvested in four different time-points (0h, 24h, 72h and 120h after treatment; for more details see Fig.1) for growth parameters, metabolic profile and gene expression analyses. We first investigated the effectiveness of the imposed treatment by analyzing the expression pattern of two important Al responsive genes in whole roots samples, which are *AtALMT 1* and *AtMATE 1* (Fig. 1). As expected, the transcript levels of *AtALMT 1* gene was significantly higher in Al treated plants in comparison with control plants (Fig. 1B). On the other hand, *AtMATE 1* gene expression was not significantly different in neither with its respective control nor with stressed plants (Fig. 1C).

Roots are the first Al target in plants and are considered the most sensible organ to this metal (Ciamporová, 2002) but leaves also are known to be responsive organs when it comes to Al stress (Mattiello et al., 2014). To show how not only roots but also Arabidopsis shoots respond to Al stress, in a carbon/biomass partitioning point of view, we analyzed the final root and shoot biomass in of the three ecotypes under control and stress environment conditions (Supp. Table S2). We found that the final shoot biomass reduced only for the Col-0 (30%) and Ws (20%) ecotypes when compared to the control but no reduction was detected in Ler, which even showing the lowest biomass among the three ecotypes, maintained its aerial parts mass in response to 5 days under Al stress. For final roots biomass, although unexpected, no significant difference was found between both treatments and ecotypes evaluated after the 5 days of experiment (Supp. Table S2).

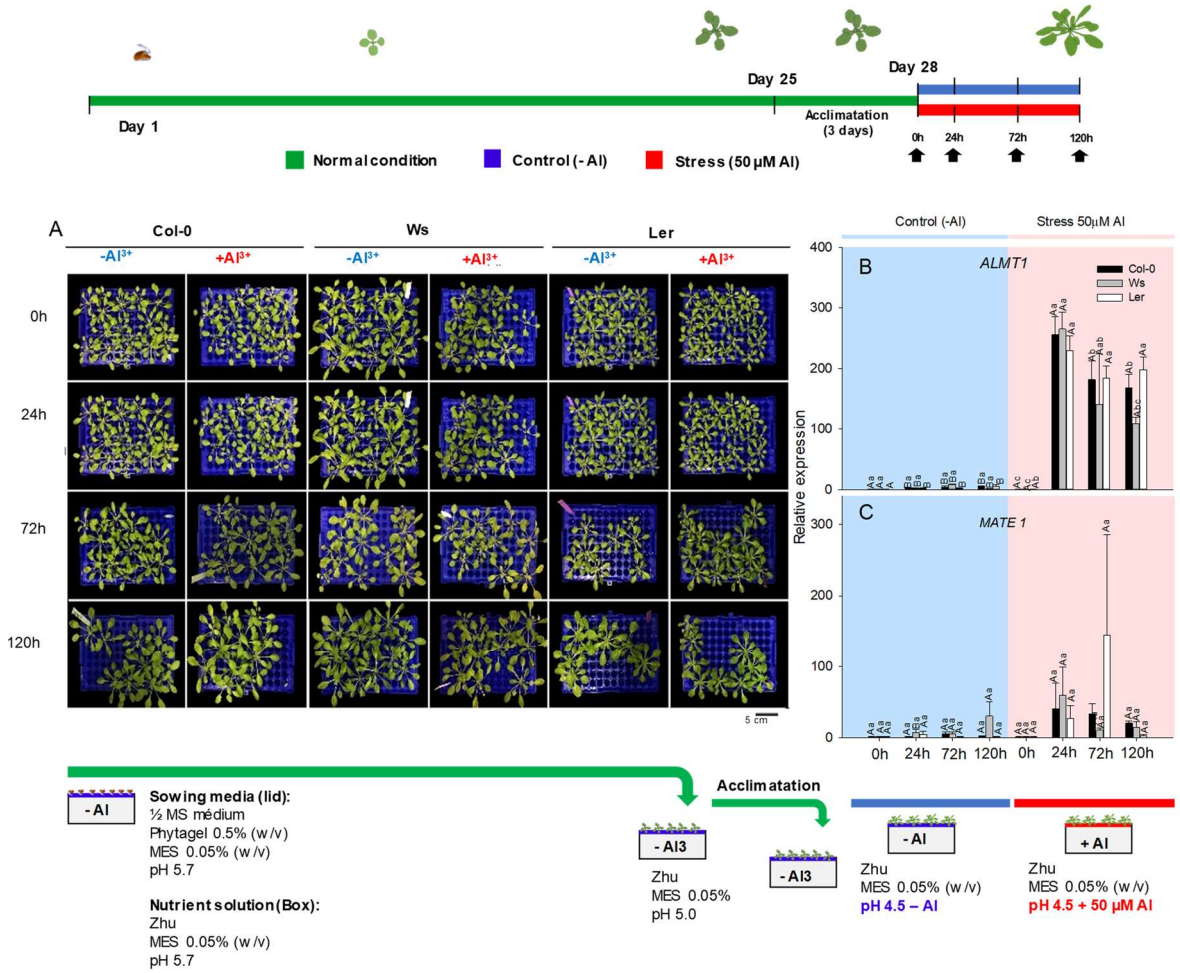


Figure 1 - General scheme representing the assembly of the hydroponic environment experiment. Seedlings were carried out directly in closed sterile boxes and maintained in ambient of high relative humidity until the 25th day for best plants selection (green bars). The most homogenous plants were selected and transferred to a new nutrient solution for acclimation in mild acid nutrient solution (pH 5.0) for three days (until 28th day) (green bars). After the acclimation period, plants were separated in two major groups. The first group being the plants conditioned at pH 4.5 without Al were called control treatment (blue bars). Finally, the second batch of plants were plants conditioned to pH 4.5 with 50 µM Al, those were called stress treatment (red bars). Both groups were concomitantly at the times: 0h, 24h, 72h and 120h. Pictures were taken during the whole experiment to show possible aerial parts phenotypes (A). To ensure that the experiment worked as expected, the expression of the major Al response genes in Arabidopsis, *ALMT1* (B) and *MATE* (C), were evaluated, with whole root samples, throughout the experiment. Values are presented as means ± SE (n=3) of independent biological replicates. Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.

The germination became similar at 30h of evaluation for Col-0 and Ws ecotypes. However, for Ler, at the same time point, it was kept higher at pH 4.5, but reduced under 100 µM of $AlCl_3$. After 5 days, the germination rate was the same for all ecotypes independently of the treatment imposed. However, the final germination was different between the ecotypes, where Col-0 and Ws showed values near 100% of germination and Ler close to 93% (Supp. Table S3).

To understand the Al effect on plant growth, we analyzed the root growth rate, final root growth, and shoot biomass (Supp. Table S3 and S4). As expected, a differential response during the seedlings establishment was observed among Col-0, Ws, and Ler (Fig. 2B and 2C and Supp. Table S3 and S4). Under 100μM of Al root growth in both Col-0 and Ws were strongly affected (Fig. 2B) whereas Ler plants showed to be less sensitive to pH and Al presence. This inhibition pattern was maintained until the last time point evaluated (Fig. 2B and Supp. Table S3 for statistics). At pH 4.5 the final root growth was inhibited in 38%, 32%, 33% for Col-0, Ws and Ler, respectively, while 25 μM was responsible for a 54% inhibition in Col-0, 53% in Ws and 42% in Ler. The higher Al concentration (100 μM Al) inhibited 71%, 76% and 58% the root final growth in Col-0, Ws and Ler respectively when compared to control pH 5.7. In contrast to Col-0 and Ws, Ler ecotype proved to be the most Al-tolerant following long-term evaluations, which despite having the lower root lengths under control conditions, presented higher root growth rates until the end of the evaluations in the higher Al concentration (Supp. Table S3). Both root growth and shoot biomass were drastically influenced by Al treatment in agar plates experiment (Fig. 2B and C). Furthermore, the pH 4.5 have drastically reduced the seedlings aerial biomass accumulation in all ecotypes (~ 40%) comparing with general control pH 5.7, and when the 25 μM Al treatment was evaluated, the shoot biomass was statistically equal to those presented by the general control, indicating a probable occurrence of the hormesis effect of Al (Barceló and Poschenrieder, 2002). The 100 μM Al treatment, however, inhibited the shoot biomass acquisition on average 40% for all ecotypes when compared to pH 5.7 control, as well as it was observed for the pH 4.5 (Fig. 2C).

Our results also showed a significant reduction in final shoot biomass in Col-0 (30%) and Ws (20%) ecotypes hydroponically grown under Al-stress conditions in comparison with control pH 4.5 without Al (Supp. Table S2); however, under the same conditions no difference was observed in root final biomass of all ecotypes (Supp. Table S2). Interestingly, Ler genotype exhibited no shoot biomass reduction (Supp. Table S2), what in agreement to previous results, ultimately classifies Ler as a more Al-tolerant ecotype in relation to the other two.

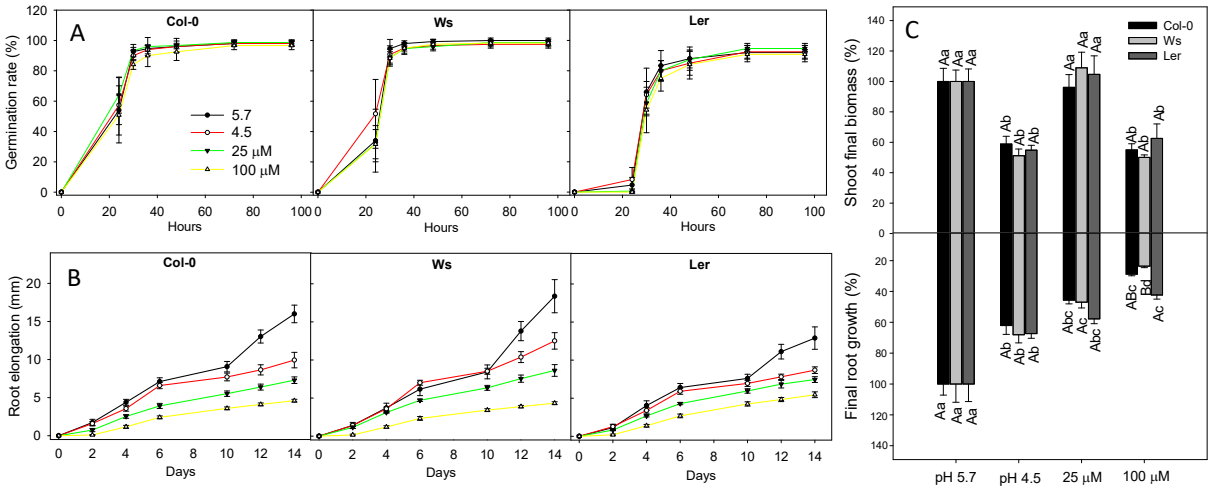


Figure 2 - *In vitro* experiment data, evaluating the three ecotypes under normal (pH 5.7), low pH (4.5) conditions and Al (25 µM and 100 µM Al^{3+}) stress environment during germination (A) and root growth (B). The relative final shoot biomass and root elongation is presented in C. The growth conditions were carried out as detailed in M&M. Data represented by, at least, four agar plates replicates with 30 seeds on each plate, for germination experiment, and 8 plants per plate in the root growth evaluation. Values are presented as means ± SE (n = 5). Different letters represent average values that were judged to be statistically different between samples ($P < 0.05$, Tukey test). Other statistics presented in Supplemental table S2.

We further evaluated the total numbers of silique, silique length and number of siliques per plant (Supp. Fig.2 and Supp. Table S2). To this end, plants were kept under hydroponic conditions until they reached the reproductive stage (Supp. Fig.2), where the control and Al treatment were pH 4.5 without Al and pH 4.5 with 100 µM Al. Additionally the nutritive solution was changed three times per week until the end of siliques formation stage. The number of siliques per plant was drastically reduced by Al, which promoted 47%, 43%, and 30% reductions for Col-0, Ws, and Ler respectively (Fig. S2 and Table S1). The silique length, which differs among the ecotypes under optimal growth conditions, was not affected by the Al treatment (Supp. Table S2). For seed weight, Ws ecotype reduced its thousand-seed-weight parameter in the Al stressed plants around 7%, moving from the heaviest in control to become an intermediate between the Col-0 and Ler under Al-stress conditions (Supp. Table S2).

3.2. Changes in Arabidopsis ecotypes metabolism under Al stress

To better understand the short and long-term Al responses of different Arabidopsis ecotypes, we further investigated the influences of Al stress on the

central metabolism. We first analyzed the absolute concentration of glucose, fructose, and sucrose (Fig. 3), starch (Fig. 4), and malate and fumarate (Fig. 5), in both shoot and roots.

Our results showed little significant changes in glucose and sucrose content on shoot tissues (Fig.3A and C). These two carbohydrates were clearly affected in Ler roots which presented reduced glucose content in stress conditions in all evaluated points (Fig.3A), and Col-0 sucrose levels in roots with a stress-induced sucrose increase. Moreover, single changes in sugars content were also observed as an increased glucose content in 72h-Col-0-stressed roots and a decreased glucose level in Ws stressed roots at 72h. Single sucrose increases were also observed in Ler stressed shoot (70%) and root (53%) at 120h (Fig. 3E-F). On the other hand, shoot fructose content was drastically influenced by Al stress in both Col-0 and Ws, whereas Ler exhibited a significant increase of 82% at 120h in treated plants (Fig.2B). Additionally, fructose content in roots was also influenced by Al-stress in all ecotypes, however, Col-0 presented an Al-induced fructose increase of 60% and 94% at 72h and 120h respectively, whereas Ws respectively reduced the fructose levels in 31% and 43% at 24h and 72h. Ler roots, on the other hand, exhibited a single decrease in fructose levels at 72h over stress (Fig. 3D).

The levels of starch were affected by the Al treatment only in shoot, which represents a modified transient starch content in Col-0 and Ws ecotypes (Fig. 4A-B). Col-0 plants accumulated 70% more starch in stressed plants at 24h compared to control, rising the difference to 165% at 72h and 70% at 120h. For Ws plants significant increased starch levels was observed only72 h in which stressed plants exhibited 95% more starch than control plants. Unlike to Col-0 and Ws, Ler not only tended to maintain its starch levels over stress, but also presented a 20% reduction at 72h in stressed plants.

The three ecotypes exhibited changes in malate (Fig. 5A) levels in shoots, with Col-0 presenting a 47% increase at 72h and 48% at 120h in response to Al compared to plants under control conditions. Ws showed a 28% increase in malate content at 24h and 26% at 120h in stressed plants, maintaining similar malate levels at 72h. Ler also increased 57% the malate levels at 120h in relation to control (Fig. 5A). Malate levels in roots (Fig. 5B) had no significant alterations in all the ecotypes, besides for a stress induced malate increase at 72h in Ws roots (Fig. 5B. In addition,

fumarate content in both shoot and roots (Fig. 5C-D) was not strongly affected by Al, however, occasional increases occurred in Col-0 shoot at 72h and Ws roots at 24h. These results suggest the organic acid metabolism is differentially affected by Al in the two evaluated tissues.

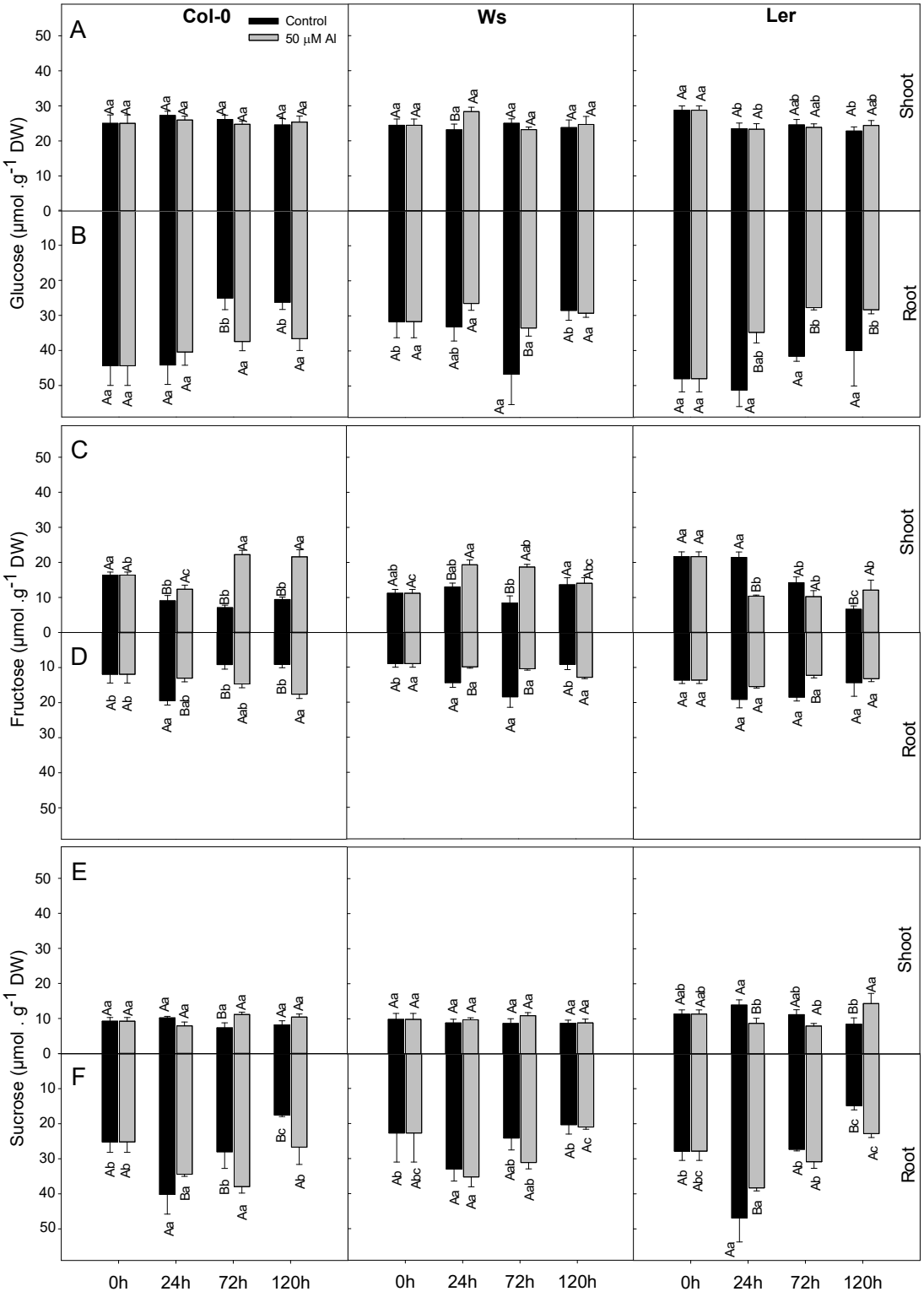


Figure 3 - Changes in soluble sugar content in Arabidopsis shoot and roots under Al stress. Glucose (Shoot – A; Roots - B), fructose (Shoot – C; Roots - D) and sucrose (Shoot – E; Roots - F) levels for five days Al stress. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different (P < 0.05, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.

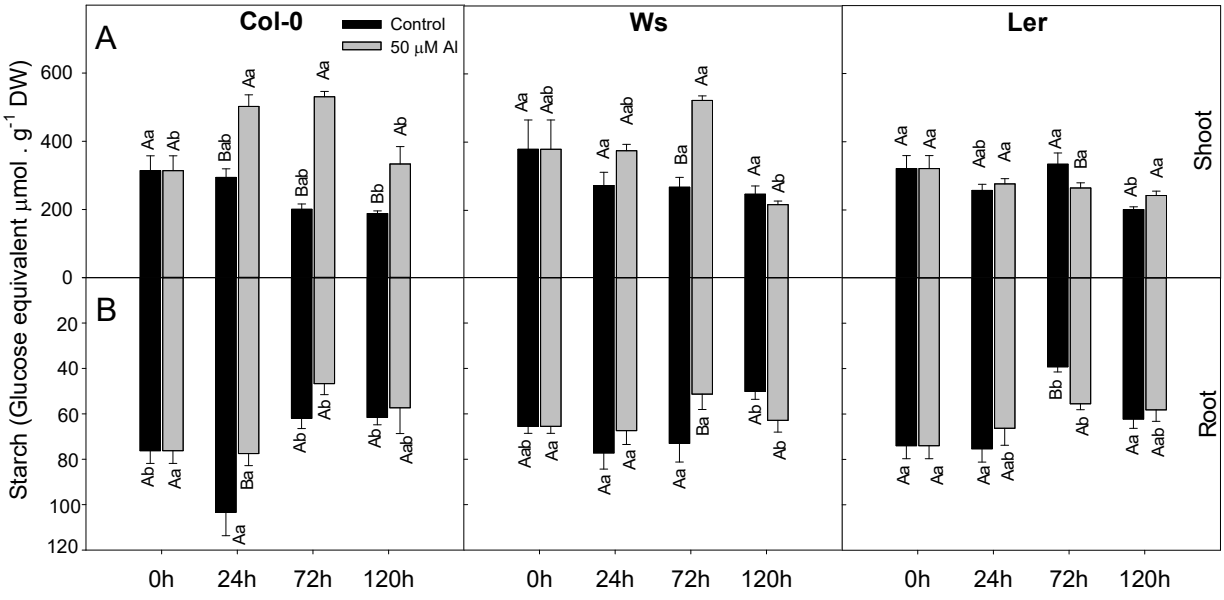


Figure 4 - Starch content variations in Arabidopsis shoot and roots under Al stress. Starch levels in the three ecotypes evaluated in shoot (A) and roots (B) for five days under Al stress. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different (P < 0.05, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.

The nitrogen-related molecules (total soluble proteins, total amino acids and chlorophylls) were also quantified in both organs (shoot and roots). The shoot total soluble protein content (Supp. Fig. S2A) did not reveal a clear response to Al treatment over time. The total protein levels in shoot did not seem to be affected by the imposed stress (Supp. Fig S3A). The total protein content (Supp. Fig S3B) observed in Col-0, Ws and Ler roots showed at least one significant change along the experiment, however these alterations did not seem to be conclusive as a response to Al stress. In this context Col-0 presented lower protein contents at 24 and 72h under Al stress, whereas higher levels were observed at 120h. Differently, Ws exhibited increased the protein levels at 24h, besides a reduction occurred at 120h in response to Al. Furthermore, the total amino acids were not highly influenced by Al stress neither in shoot (Supp. Fig.S3C) or roots (Supp. Fig.S3D), except for a two-days-decrease in amino acids content presented by Al-stressed Ws shoot at 72h and 120h, as well as a single reduction in Ler shoots at 72h in response to the stress.

Additionally, we evaluated chlorophyll *a* and chlorophyll *b* contents (Supp. Fig. S4A-B), and chlorophyll *a*/*b* ratio (Supp. Fig. S4C). In general, the chlorophyll content did not seem to be influenced by Al in the early time points, at least the ecotypes did not show significant alterations until the 72h of stress. However, slight changes occurred specially in response to Al for Ws which increased chlorophyll a (Suppl. Fig. S4A), decreased the chlorophyll b (Suppl. Fig. S4B), what ultimately increased *a/b* ratio (Suppl. Fig. S4C) at 120h. Additionally, Ler proportionally increased both chlorophylls *a* and *b* in response to Al, what maintained the *a/b* ratio (Suppl. Fig. S4A-B-C). This data suggests that chlorophylls might be differently affected by Al depending on the studied ecotype however, it seems to respond better in a much longer time of Al exposure in Arabidopsis.

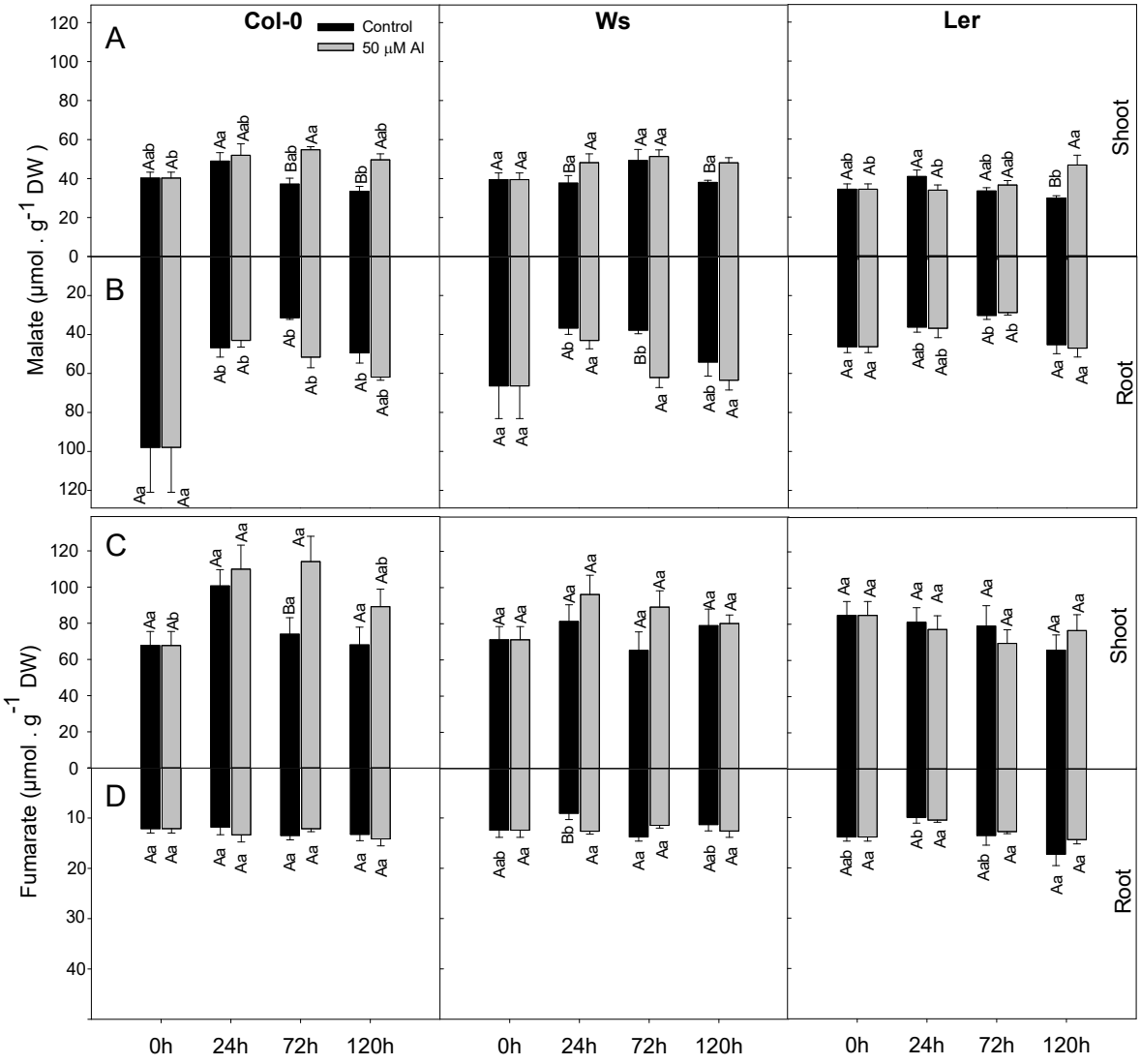


Figure 5 - Organic acids alterations in shoot and root of Arabidopsis ecotypes in response to Al stress. Malate (Shoot – A; Roots - B) and fumarate (Shoot – C; Roots - D) levels in the three ecotypes evaluated in both tissues (*i.e.* leaves and roots) for five days Al stress. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.

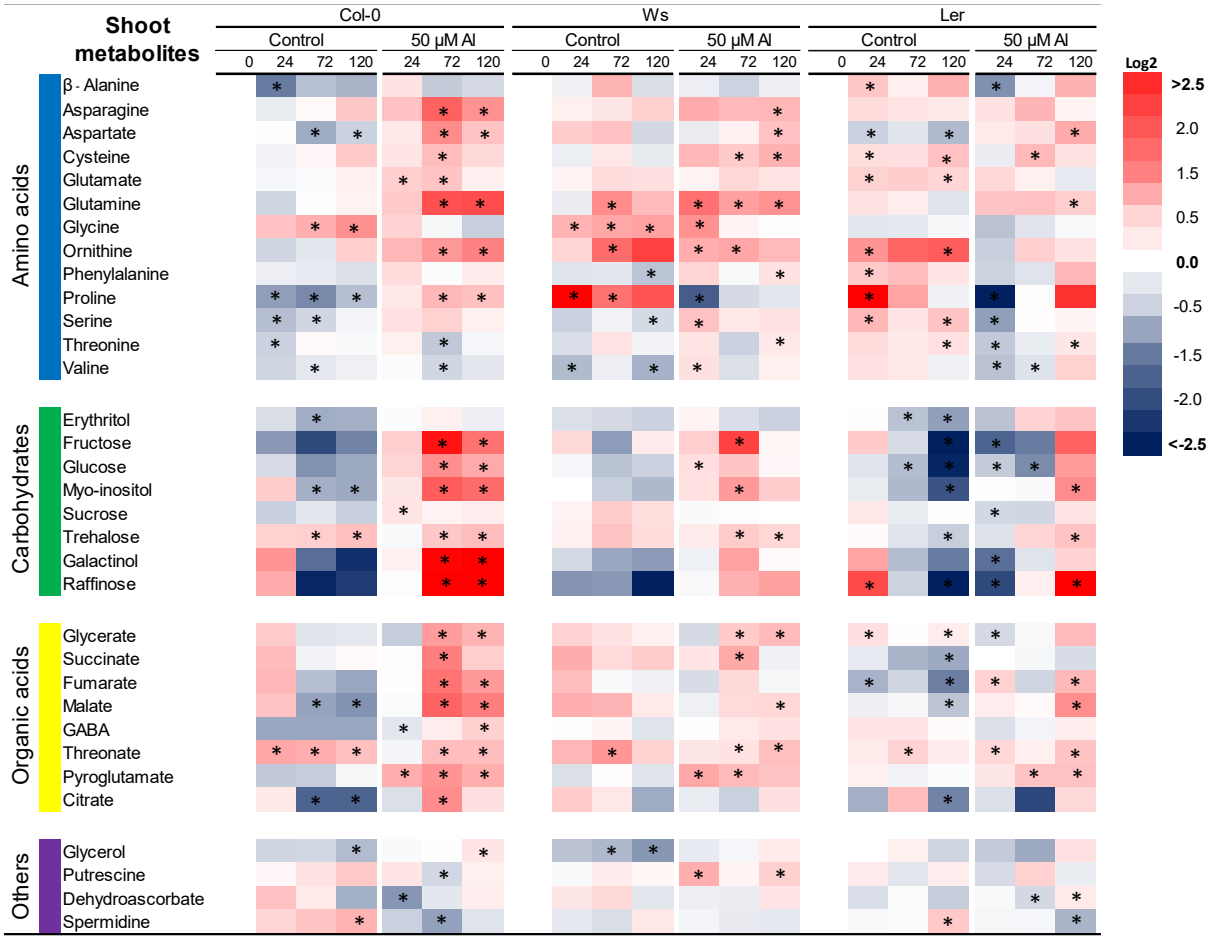


Figure 6 - Heat map representing changes in shoot metabolites in Arabidopsis ecotypes during the whole experiment, analyzed by GC-MS. Control and treatment samples were normalized differently, being control data normalized by "timepoint 0h", and treatments were normalized by their respective control time point. According to the lateral scale, red color intensity represents increases in the metabolite levels, and blue intensity shows reduction levels. Values are presented as means ± SE (n=4). Asterisks in controls (24h, 72h e 120h) indicate that these values were significantly different ($P < 0.05$) when compared with "timepoint 0h". In treatment plots, asterisks indicate values significantly different ($P < 0.05$) from their respective control timepoint. Both determined by Student's *t* test.

In order to obtain a better characterization of the Al effects on the central metabolism in the studied ecotypes, we next decided to extend our study to the major pathways of the primary metabolism by using an established gas chromatography-mass spectrometry (GC-MS) based metabolite profiling. This approach allowed us to identify 33 metabolites in shoot (Fig. 6) and 41 in roots (Fig. 7) samples. Initially, the metabolite data was normalized in two different ways, where controls were compared

to the time 0h, in order to understand the natural metabolite variation in hydroponics conditions. On the other hand, treatments were compared with their respective time controls, in order to demonstrate the variation caused by the stress in the respective point. The identified metabolites were classified in four major groups namely amino acids, carbohydrates, organic acids and others (metabolites that did not fit into the previous groups). We identified 33 metabolites, which presented basal fold-changes in control conditions, which in general tended to decrease over the time after treatment. Additionally, different Al induced response in relation to each ecotype was clearly presented. Regarding the stress conditions, the amino acids levels were differentially changed in all ecotypes shoot tissues, where Col-0 and Ws presented an increasing pattern for some primary products of nitrogen assimilation (asparagine, aspartate, glutamine) (Lam, 1995; Coruzzi, 2003) as well as increases in ornithine. Ler, however presented only few amino acids increase in response to Al for aspartate, glutamine. Cysteine, which is considered toxic in higher levels in plants (Hildebrandt et al., 2015) commonly increased in all ecotypes. Regarding the carbohydrates levels no further changes was observed in control conditions except for significant decreases in Ler ecotype. Additionally, Al stress promoted considerable increases in carbohydrates levels specially for Col-0 and Ws, from which Col-0 was clearly more affected. Ler shoots on the other hand, responded to Al stress presenting an early decrease in carbohydrates levels with few increases (at 120h) for myo-inositol, trehalose and raffinose. Finally, all the ecotypes showed to commonly increase some organic acids such as pyroglutamate, threonate, malate and fumarate, which are involved in glutamate metabolism, ascorbate degradation and TCA cycle (malate and fumarate) intermediates (Fernie et al., 2004; Kumar and Bachhawat, 2012; Truffault et al., 2017).

Roots GC-MS analysis was also performed with a total of 44 compounds identified (Fig. 7) and the data was normalized as previously described for shoot data. Interestingly, the metabolites behavior under normal and Al-stressed condition was similar to that presented by shoot analyses, however with lower fold-change intensities. In roots over control a time-dependent reducing pattern was observed for Col-0 and Ws amino acids levels, what was not true for Ler, which sowed significant increases for six different amino acids (alanine, glutamine, isoleucine, methionine, proline and valine). Additionally, it was possible to observe a common Al-induced

amino acid increase for Col-0 and Ws glutamine, methionine, proline, serine and threonine levels, while Ler was poorly affected by the stress with increased levels of β-alanine, proline, threonine and valine.

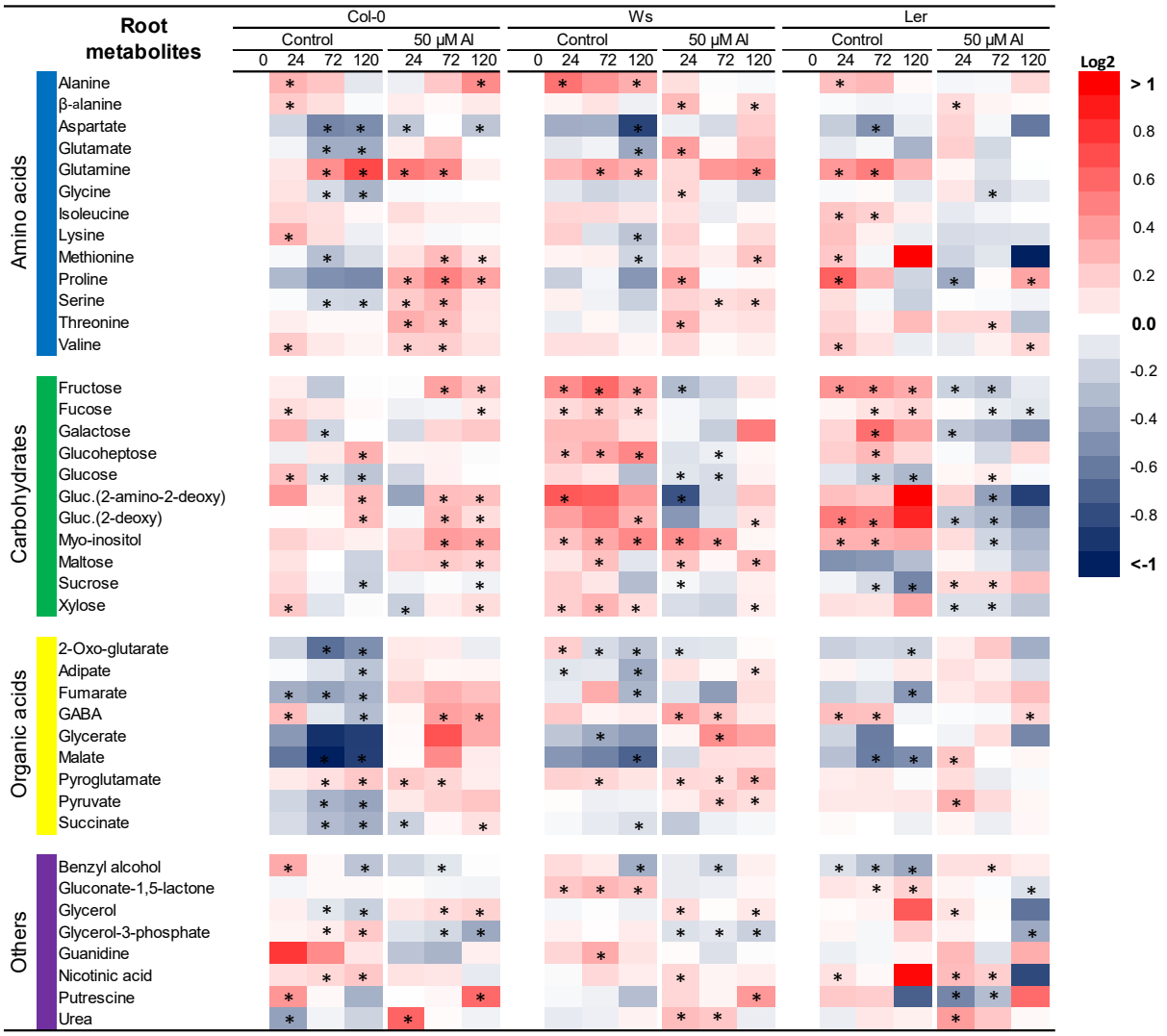


Figure 7 - Heat map representing changes in root metabolites in Arabidopsis ecotypes during the whole experiment, analyzed by GC-MS. Control and treatment samples were normalized differently, being control data normalized by "timepoint 0h", and treatments were normalized by their respective control time point. According to the lateral scale, red color intensity represents increases in the metabolite levels, and blue intensity shows reduction levels. Values are presented as means ± SE (n=4). Asterisks in controls (24h, 72h e 120h) indicate that these values were significantly different ($P < 0.05$) when compared with "timepoint 0h". In treatment plots, asterisks indicate values significantly different ($P < 0.05$) from their respective control timepoint. Both determined by Student's *t* test**.**

Roots were clearly affected by Al regarding carbohydrate levels with significant increases observed for Col-0 which increased most of the identified compounds of this group over Al stress (except for galactose, glucoheptose, glucose and sucrose), while Ws presented few increases in myo-inositol, 2-deoxyglucose, maltose and

xylose levels. Interestingly, Ler showed early (24h and 72h) decreased levels for almost all carbohydrates except for the transport sugar sucrose which significantly increased in this ecotype's roots, suggesting higher respiration events in response to Al stress in this tissue. Finally, the organic acids were indeed affected by Al in roots, however the only common metabolite increased by Al among the three ecotypes was the ɣ-amino butyric acid (GABA). Col-0 and Ws exhibited more organic acids increases then Ler. Ultimately, this data suggests that not only in shoot but also in roots the TCA cycle is affected by Al stress, as well as clear impacts on N (nitrogen) and carbohydrate metabolism.

Once few changes of root TCA cycle intermediates as malate, succinate, pyruvate in some ecotypes were correlated with strong differences in root carbohydrate content we decided to run a new experiment to determine root respiration (Supp. Fig. S5) evaluating the Al effect over whole root respiration in order to infer about the TCA cycle involvement with the above-mentioned parameters. Interestingly, Col-0 and Ws roots did not show major differences between the control and stress treatment at any evaluation time except for a high respiration presented by Ws roots at the fifth day under both conditions. In contrast, Ler initially behaved similarly to the other two ecotypes, however, at 72h and 120h it is possible to observe a high respiration rate for plants exposed to the Al stress, exhibiting lower carbohydrate and organic acids level, which could be consumed and not accumulated, respectively, in the sense of quickly deal with the stress.

3.3. Al stress responsive genes are differentially modulated in shoots and roots

Many reports have shown that Al tolerance involves several specific Al responsive genes such as *ALMT1* and *MATE1* in Arabidopsis (Fig. 1), thus we performed a time-course of gene expression analysis of two transcription factors, *WRKY46* which represses *ALMT1* expression (Ding et al., 2013) and *STOP1* involved in the Al-induced expression of *ALMT1* as well as *MATE1* (Fan et al., 2016). In addition, we also analyzed the expression pattern of sucrose transporter (*SUC2*) in order to possibly observe differences on an Al-induced sucrose transport from shoot to roots and likely understand distinct carbon allocation patterns. Also, two ROS responsive genes, namely *GST1* and *ZAT12*, in both shoot and root tissues under

normal and stressed condition (Fig.8). Surprisingly, the qRT-PCR revealed no significant alteration in the total transcriptional level of any chosen genes neither in shoot or roots when both treatments were compared or even along the experiment time. Our results suggest that the expression of *STOP1* and *WRKY46* genes in shoot is not Al-influenced as it was expected in root organs subjected to pH and Al stress, respectively. In roots, although it was expected the Al-induced repression of *WRKY46* (Ding et al., 2013) or the low pH inducing *STOP1* expression (Sawaki et al., 2009), the accuracy of the expression analyzes was probably compromised due (i) a specific small root region where this genes are responsive (root tips) and (ii) probable low transcript abundance of these genes. Moreover, Al stress neither promote a linear response together with ROS responsive genes nor seemed to be important to promote expression of *SUC2* gene. When we evaluated ROS responsive genes as well as the sucrose transporter, they seemed not to be Al responsive.

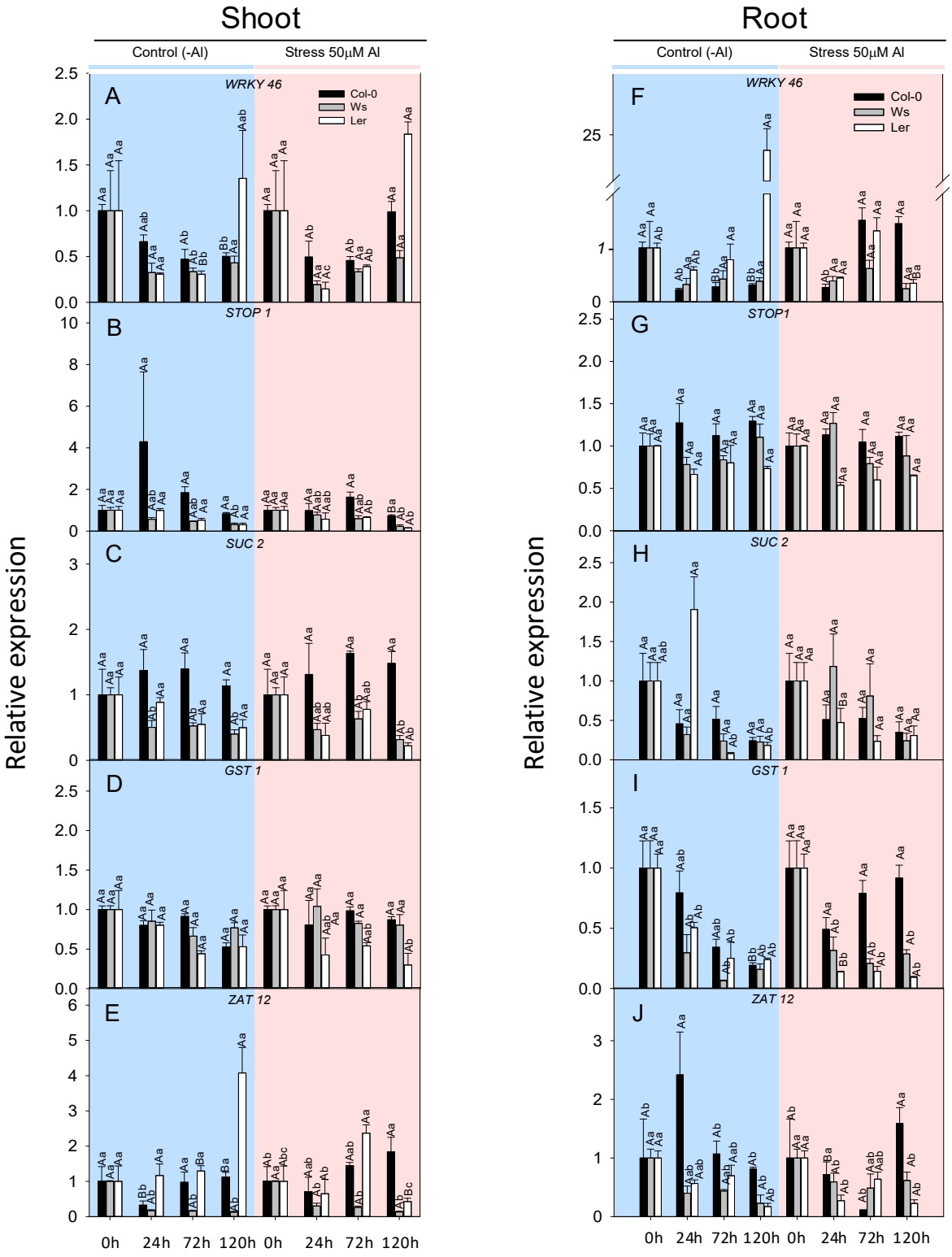


Figure 8 - Shoot and Root relative transcript responses of Al-related genes in response to Al treatment. Five genes were evaluated in control (pH 4.5 - in blue) and stress environment (50 μM Al - in red) in both tissues (*i.e.* shoot and roots), being two transcription factors involved in aluminum responses: *WRKY46* (shoot A; root F) and *STOP 1* (shoot B; root G); a sucrose transporter gene *SUC2* (shoot C; root H); two ROS responsive genes: *GST1* (shoot D; root I) and *ZAT12* (shoot E; root) in all Arabidopsis ecotypes. The y-axis values represent the relative expression levels which were calculated using the 2-ΔΔCT method (see details M&M section). All values, control and stress treatment samples were normalized by the "timepoint 0h". To normalize gene expression was used the constitutively expressed gene *Actin* for all samples, under a better concentration according

standard curve primers. Values are presented as means ± SE (n=3) of independent biological replicates. Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 μM Al), and lower-case letters demonstrate statistical differences along harvesting times.

4. DISCUSSION

Following a previously developed hydroponic system, here an hydroponic Al-assay was properly adapted which allowed us to cultivate different Arabidopsis ecotypes on both short and long-term experiments with Al. By monitoring both the growth and final seed yield production coupled with the metabolic and physiologic mechanisms associated with Al tolerance, we were able to demonstrate that the differential Al tolerance is ecotype dependent and that metabolic and physiologic acclimation responses likely occur contributing to the maintenance of plant growth in response to Al stress.

4.1. Ler ecotype is most capable to deal with Al stress

To prove the effectiveness of the Al treatment, we first evaluated the expression pattern of two Al-responsive genes, *AtALMT1* and *AtMATE1,* which *are* responsible for malate and citrate exudation, respectively (Liu et al., 2009). Although *AtALMT1* and *AtMATE1* together confer full expression of Arabidopsis Al tolerance (Liu et al., 2009), *AtALMT1* is clearly the major determinant of Al-tolerance (Hoekenga et al., 2006). In our study, as expected, *AtALMT1* presented higher transcript levels under Al treatment when compared with non-treated plants (Fig. 1B). On the other hand, no significant difference was observed in *AtMATE1* transcript levels (Fig. 1C), which was most likely due the fact that the expression analysis was derived from whole roots samples. Therefore, higher transcript levels of *ALMT1* in relation to *MATE1* was in good agreement with previous observation (Liu et al., 2009); moreover, root malate and citrate exudation are spatially concentrated at the root tips (Ryan et al., 1993; Sivaguru and Horst, 1998; Kochian et al., 2015), thus a probable dilution in transcript abundance may have occurred for *MATE1* (Fig. 1C). Thus, the high relative expression of *AtALMT1* for all ecotypes (Fig. 1B) assured that the Al stress certainly occurred. High Al-activated malate exudation as well as *AtALMT1* transcripts abundance are closely associated (Kobayashi et al., 2007; Liu

et al., 2009; Sawaki et al., 2009; Liu et al., 2012). Although we did not evaluate the root tip exudation it is reasonable to assume that Al-activated malate exudation occurred in all studied ecotypes. It has been previously shown that Col-0 presented highest Al-activated malate efflux rate compared to Ler ecotype (Hoekenga et al., 2003). In addition, Hoekenga et al. (2006) estimated Al tolerance in nine Arabidopsis ecotypes by using a short-term root growth approach coupled with Al-activated malate efflux rate. It was shown that Col-0 present high exudation rate and higher root growth as therefore considered the most Al tolerant ecotype, followed by Ws and Ler, the last one presenting the lowest root growth and Al-activated malate efflux rate. Surprisingly, we found that Ler was the only ecotype capable of maintaining the high *ALMT1* transcript levels (Fig. 1B) in Al-treated roots. Our results demonstrate that Ler is most likely able to sustain *ALMT1* expression with lower malate exudation which allows this ecotype to handle the Al stress for longer times without wasting carbon molecules. This result together with the lower relative decrease in growth (Fig. 2; Supp. Tables S2-3-4) and yield parameters (Supp. Table S2) measured in long-term experiments indicates that Ler plants were able to better cope with Al than Col-0 and Ws under our experimental condition.

Here, we compared three Arabidopsis ecotypes regarding their Al tolerance which has been previously performed suggesting Ws as the most Al-tolerant, followed by Col-0 and Ler considered as the most Al-sensible (Toda et al., 1999). Nevertheless, we focused in further advancing this previous observation and possibly link the different growth parameters between the ecotypes under Al-stressed conditions with main metabolic shifts. The three distinct ecotypes were contrasted following different experimental approaches involving Al stress. We evaluated in agar plates the effect of Al on all developmental stages beginning with germination (Fig.2A; Supp. Table S3) root growth rate (Fig.2B; Supp. Table S3), final root length (Fig.2C; Supp. Table S4), final shoot biomass (Fig.2C; Supp. Table S4). In addition, we analyzed biomass increment in both root and shoot in 4-week-old plants kept on hydroponic system and the final crop yield production parameters (Supp. Table S2). Although no differences between ecotypes were found for final germination assays (Fig. 2A; Supp. Table S3) and final root biomass (Hydroponic environment Supp. Table S2), all the other evaluated traits showed significant reductions in response to Al. It is interesting to notice that final root growth was not affected in any ecotype

under Al stress conditions, what was probably expected due to the root-stressing environment occurring in control, such as low pH (pH 4.5), known to be inhibitory for root growth (Liang et al., 2013; Shavrukov and Hirai, 2016). Ler presented the lowest absolute values for several traits including in final seed germination (Supp. Table S3), root growth (Supp. Table S3) and shoot biomass (hydroponic environment - Supp. Table S2; agar-plate experiment - Supp. Table S4) and yield (Supp. Table S2); remarkably, for all this traits Ler also presented the lowest relative reductions, as observed in Figure 2C. In addition, this ecotype also presented only 30% yield reduction in comparison to the control, whereas Col-o and Ws were characterized by 47% and 42% yield reduction, respectively (Supp. Table S2).

It is also worth mentioning that Al not always induces negative effects in plants (Barceló and Poschenrieder, 2002; Bojórquez-Quintal et al., 2017), but also can promote growth depending of concentration, time of exposure and the studied genotype. Accordingly, Al induced shoot biomass (Fig. 2C) for all the ecotypes compared with the pH control (pH 4.5) when in low concentration (25 μM), but still culminated with reduced root length when also compared to pH 4.5. Collectively, these results suggest an imbalanced carbon partitioning between shoot and roots in response to Al conditions. Altogether, the results obtained here led us to characterize the Ler ecotype as the most capable to deal with Al stress. Although Col-0 and Ws are naturally more robust and productive under no stressful conditions, they also showed to be more susceptible to Al in long-term experiments.

4.2. Al stress lead to differential metabolic response in Arabidopsis ecotypes

To elucidate the connection between Al stress and metabolic alterations we further conducted a detailed metabolic characterization in both shoot and root tissues. Since a range of plant species evolved mechanisms that enable growth on high toxic Al soils mediated by Al-induced OA release detoxification in the rhizosphere, we decided to analyze the metabolic adjustments in both root and shoot to identify the main changes on the central metabolism which is directly involved in the growth maintenance.

Even though soluble sugars, which act on the supply of carbohydrates from source organs to sink ones (Rosa et al., 2009), are highly sensitive to environmental

stresses, shoot glucose content was not affected by Al stress in any ecotype (Fig. 3A-B). However, in roots of Ler, reduced glucose levels in response to Al stress was observed in all evaluated days (Fig. 3B). This indicates that glucose consumption is likely occurring at high levels during the glycolytic respiratory pathway in roots, which is in good agreement with the higher O_2 consumption rate (Supp. Fig. 5). In a different way, fructose content was affected in Col-0 and Ws in both shoot and roots (Fig. 3C-D) with significant increases in Col-0 and Ws shoot in response to Al treatment. Moreno-Alvarado et al., (2017) has proposed that soluble sugars are a possible signaling response to Al-tolerance in rice leaves. It seems reasonable to assume that under our conditions Col-0 plants (and to a lower extent in Ws) this signaling process involving increased fructose levels might have also occurred, however it might not be related to a higher tolerance in Arabidopsis. Furthermore, it seems that Al treatment affected the carbon use by diminishing the capacity of carbon use which is in close agreement with shoot starch accumulation (Fig. 4) that is negatively correlated with biomass gain in Arabidopsis (Sulpice et al., 2009). In addition, Ler did not present accumulation in shoot starch content in response to Al (Fig. 4) and might be associated to the absence of reductions in shoot growth (Supp. Table S2). Nevertheless, the fructose content in roots was characterized by different patterns among the ecotypes (Fig. 3D). Col-0 was the only ecotype to present increases on fructose content at 72h and 120h in stressed roots (Fig 3D), which might be closely related with a concomitant increase of sucrose in roots following 72h and 120h in presence of Al (Fig. 3F). This is explained, at least partially, by a higher sucrose translocation capacity, in agreement with the increased glucose level (Fig. 3B) at 72h and a maintenance of glucose levels at 120h. On the other hand, Ws and Ler presented no Al-induced increases of fructose in roots, but rather reductions at 24h and 72h for Ws and reduction for Ler at 72h, which seems to not be linked to reductions in the levels of sucrose in roots of these ecotypes (Fig. 3F).

Increases in OAs content was observed in shoot tissues due to the Al treatment in Col-0 (72h and 120h, ~47% in both times), in Ws (24h and 120h, ~27% in both times) and Ler (57% at 120h). Shoot malate accumulation in Col-0 and Ws could be explained partially by a reduction in the TCA cycle operation or biosynthetic reactions of amino acids, for instance (Ishizaki et al., 2006; Araújo et al., 2010). Even though no further differences occurred for total amino acids content (Supp. Fig. S3C-

D), Col-0 and Ws ecotypes presented significant increases in specific amino acids in shoots following our metabolite profile (Fig. 6). This reduced TCA cycle operation in shoots of both Col-0 and Ws probably led to the increased fructose levels (Fig. 3C) and the subsequent rise in starch (Fig. 4A). If that is true, a possible negative effect of reduced TCA cycle operation on the photosynthetic reaction of Col-0 and Ws may also have occurred (Finkemeier and Sweetlove, 2009). Notably, the results obtained by Ler are not fully explained by this assumption that only explains the increased malate levels (57%) at 120h (Fig. 5A), coupled with the increase in fructose levels (82%) in response to Al (Fig. 3C), but it does not explain the absence of changes in starch accumulation at 120h in Ler shoots (Fig. 4A). Fumarate levels (Fig. 5C-D), differently from malate, were virtually invariant indicating that this OA is most likely not responsive to Al stress. Notwithstanding, the possible impacts of Al on photosynthesis remains to be elucidated. Thus, further analyses concerning dark respiration combined with diel fluctuation of starch would expand our understanding on the impact of Al over not only starch degradation and growth, but may also provide new insights about TCA cycle operation in response to Al.

Although we have postulated the importance of changes in photosynthetic reactions in response to Al, no clear pattern of changes in N-containing compounds including total proteins, total amino acids and chlorophyll content were observed. We cannot ascertain whether the time or the Al concentration (or both) were severe enough to impact these compounds. However, it seems reasonable to anticipate that to cope with Al, Arabidopsis plants seem to maintain the levels of such compounds most likely to support growth.

A detailed understanding of the metabolic changes following Al stress is still lacking. Nevertheless, our metabolite profile approach showed significant increases in the levels of several amino acids, carbohydrates and organic acids in shoots of both Col-0 and Ws. We observed that higher levels of metabolites are usually found in Col-0 and Ws whereas lower levels in Ler shoots. This feature is compatible with soluble sugars (Fig. 3), specially for fructose (Fig. 3C) which accumulated in Col-0 and Ws shoots. We posit that this accumulation might be due to a lower carbon use and respiratory capacity, which probably, led to starch accumulation in both ecotypes under Al-stress (Fig. 4). Notably, the observed increase in amino acids in shoots (Fig. 6) is not necessarily linked to a higher protein turnover or degradation. Probably this

raise in association with an Al-induced amino acid interconversion and accumulation of specific amino acids in Col-0 and Ws works most likely as a signal of carbohydrate starvation (Hildebrandt, 2018), (since carbohydrates appears not to be fully consumed). Accordingly, the accumulation of amino acids and carbohydrates was previously observed in shoot and roots of different maize genotypes under Al (Khan et al., 2000) and it was related to Al-tolerance. In contrast, a decreasing pattern was observed for fructose, glucose, sucrose (in a lower extend) as well as for galactinol and raffinose at 24h in shoots of Ler (Fig. 6). Thus, if carbohydrate reductions over the time is a response of higher carbon use (Sulpice et al., 2009), that likely occurs with higher respiration capacity and ultimately, lower starch accumulation (Fig. 4), that should, once more, explain the shoot biomass maintenance in Ler under Al stress (Supp. Table S2). Although many reports relate carbohydrate or amino acids increase in shoot and roots with as non-specific physiological mechanism linked to probable high Al tolerance in maize, rice and sorghum (Cambraia et al., 1983; Khan et al., 2000; Moreno-Alvarado et al., 2017) it does not seems to be true for Arabidopsis as presented here. It does, however, mean that considerable work is still required to achieve mechanistic understanding of Al tolerance in land plants.

Interestingly, roots metabolites (Fig. 7) seemed to follow a similar pattern observed in shoots (Fig. 6), where Col-0 exhibited increasing in amino acids, carbohydrates, and in a lesser extension, in organic acids in response to Al. Col-0 plants exhibited one single increase in total amino acids content at 72h in stressed plants (Supp. Fig. S3), that further explains the concomitant on methionine, proline, serine, threonine and valine levels after 72h in presence of Al (Fig. 7). Similarly, Ws showed increasing levels of specific amino acids (without increasing total amino acid - Supp. Fig. S3C), carbohydrates and organic acids under Al-stress conditions. By sharp contrast, clear pattern of reductions for most metabolites under Al-stress conditions was observed for Ler. Nevertheless, the increased levels of sucrose only presented by Ler roots (Fig. 7), is interesting once it might represent higher sucrose translocation, which is probably inhibited by Al in Col-0 and Ws ecotypes possibly through an Al-induced inhibition of sucrose transporters in root tips (Sameeullah et al., 2013; Kariya et al., 2017). Thus, this higher sucrose content in Ler roots might represent a possible increased carbohydrate translocation providing higher levels of substrate for root respiration (Supp. Fig. S5) and root growth maintenance (Ruan,

2014). Moreover, sucrose is highly related to Al-stress alleviation (Kariya et al., 2017) helping cell elongation and serving as a carbon source for synthesis of cellular components and ATP.

The expression pattern of two pH and Al-related transcription factor *AtWRKY46* (Fig. 8A-F) and *AtSTOP1* (Fig. 8B-G) were further investigated and compared with the possible changes in carbon metabolism. Although *WRKY46* was previously related to other processes in plants such as stomatal movements (Ding et al., 2014) and pathogen responses (Hu et al., 2012), as well as *STOP1* was also related with homeostasis and pH-regulating metabolism (Sawaki et al., 2009) , we did not observe any further relation between their expression pattern and the metabolic changes neither in shoot or roots. To further investigate the ecotypes capacity to deal or rapidly respond to oxidative stress, two ROS responsive genes, namely *GST1* (Fig. 8D-I) and *ZAT1* (Fig. 8E-J), were assayed in both shoot and roots but no linear pattern could be observed or linked with ROS staining (Supp. Fig. S1). Sucrose was previously reported to be an Al-alleviator in tobacco plants when sucrose transporter (*NtSUT1*) was overexpressed (Kariya et al., 2017). To further understand the relation between the carbon synthesized in shoot and the sucrose levels translocated to roots, we evaluated the expression of *SUC2 (*Fig. 8C-H), a sucrose transporter gene, which presented enhanced expression pattern over stress conditions, such as in drought (Durand et al., 2016). Nevertheless, no direct relation between the expression pattern of *SUC2* and Al stress was found. In light of the results showing virtually no changes in the expression pattern of Al-related genes, coupled with the significant metabolic changes observed in both shoots and roots, further cell specific studies are clearly required to fully understand the importance of genes related with sucrose transport (or phloem loading) in Arabidopsis plants in order to verify a possible mechanism of Al-stress relieve.

5. CONCLUDING REMARKS

To summarize, we have focused in understanding the differential response of three ecotypes of *A. thaliana* and how and to which extent the different Al-stress tolerance was related to physiological and metabolic mechanisms. Collectively, the results obtained allowed the proposition of a new Al tolerance classification between these three ecotypes (Col-0, Ws and Ler) regarding short-term and long-term Al-stress analyses. Moreover, the complexity of Al impact and responses in plants was initially confirmed with the high Al-induced expression of the well-known *ALMT1* gene in all the studied ecotypes added to a complex metabolic response in both shoot and root organs. Although Ler is seemingly less productive regarding biomass and yield terms, this genotype displayed relatively lower reductions in biometric parameters in response to Al stress, what led us to classify Ler as the most Al tolerant ecotype in relation to Col-0 and Ws. The higher capacity to deal with Al was initially accompanied by lower soluble sugar accumulation and possible higher starch consumption during dark respiration. Moreover, higher respiration rates in roots can be also linked to higher carbohydrates consumption in roots of Ler. By contrast to the situation observed for Col-0 and Ws ecotypes, Ler did not exhibit Al-induced increases in amino acids, carbohydrates or organic acids contents in both shoot and root tissues.

When considered together, the results of this work demonstrate that the overall responses to Al stress are more complex than previously thought. In addition, the results obtained here also uncovers a sophisticated mechanism of metabolic interplay between shoots and roots that precisely modulate Al tolerance in Arabidopsis. Further analyses are necessary to elucidate whether higher organic acids (*i.e.* malate) efflux is the main metabolic cost for less Al-tolerant ecotypes (Col-0 and Ws) in long-term experiments. Furthermore, our data demonstrated that carbohydrates and starch turnover (synthesis and degradation) dynamics, modulate by photosynthesis and dark respiration, are likely of crucial significance in response to Al availability. That being said, additional studies on the relationship between photoperiod and Al responses, once the OAs exudation and root growth seems to be also modulated by light-period, are necessary. Finally, the present study reinforces the Al-stress as a complex trait for plants and by the application of sensitive flux

profiling approaches (Batista-Silva et al., 2016) and the adaptation of cell specific profiling (Mustroph et al., 2009) is likely to enable us to pursue new avenues to understand the complex networks governing the function of primary metabolism within Al tolerance in land plant. Finetuning these interactions provides potential new strategies for enhancing plant Al tolerance and crop yield on acidic soils.

6. REREFENCES

Aftab T, Khan MMA, Idrees M, M. Naeem M (2010) Effects of aluminium exposures on growth, photosynthetic efficiency, lipid peroxidation, antioxidant enzymes and artemisinin content of *Artemisia annua* l. J Phytol **2**: 23–37

Araújo WL, Ishizaki K, Nunes-Nesi A, Larson TR, Tohge T, Krahnert I, Witt S, Obata T, Schauer N, Graham I a, et al (2010) Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. Plant Cell **22**: 1549–63

Arvidsson S, Kwasniewski M, Riaño- DM, Mueller-roeber B (2008) QuantPrime – a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics **9**: 1–15

Barceló J, Poschenrieder C (2002) Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: A review. Environ Exp Bot **48**: 75–92

Batista-Silva W, Daloso DM, Araújo WL, Nunes-Nesi A, Fernie AR (2016) Can stable isotope mass spectrometry replace radiolabelled approaches in metabolic studies? Plant Sci **249**: 59–69

Bojórquez-Quintal E, Escalante-Magaña C, Echevarría-Machado I, Martínez-Estévez M (2017) Aluminum, a friend or foe of higher plants in acid soils. Front Plant Sci **8**: 1–18

Bradford MM (1976) A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248–254

Cambraia J, Galvani FR, Estevão MM, Sant'Anna R (1983) Effects of aluminum on organic acid, sugar and amino acid composition of the root system of sorghum (Sorghum bicolor L. Moench). J. Plant Nutr. 6:

Cavalcanti JHF, Esteves-Ferreira AA, Quinhones CGS, Pereira-Lima IA, Nunes-Nesi A, Fernie AR, Araújo WL (2014) Evolution and functional implications of the tricarboxylic acid cycle as revealed by phylogenetic analysis. Genome Biol Evol **6**: 2830–2848

Ciamporová M (2002) Morphological and structural responses of plant roots to

aluminum at organ, tissue, and cellular levels. Biol Planatarium **45**: 161–171

Coruzzi GM (2003) Primary N-assimilation into amino acids in Arabidopsis. Arab B **2**: e0010

Cruz CD (2013) GENES - a software package for analysis in experimental statistics and quantitative genetics. Acta Sci - Agron **35**: 271–276

Delhaize E, Gruber BD, Ryan PR (2007) The roles of organic anion permeases in aluminium resistance and mineral nutrition. Fed Eur Biochem Soc **581**: 2255–2262

Ding ZJ, Yan JY, Xu XY, Li GX, Zheng SJ (2013) WRKY46 functions as a transcriptional repressor of ALMT1, regulating aluminum-induced malate secretion in Arabidopsis. Plant J **76**: 825–835

Ding ZJ, Yan JY, Xu XY, Yu DQ, Li GX, Zhang SQ, Zheng SJ (2014) Transcription factor WRKY46 regulates osmotic stress responses and stomatal movement independently in Arabidopsis. Plant J **79**: 13–27

Durand M, Porcheron B, Hennion N, Maurousset L, Lemoine R, Pourtau N (2016) Water deficit enhances C export to the roots in A. thaliana plants with contribution of sucrose transporters in both shoot and roots. Plant Physiol **170**: 1460–1479

Eekhout T, Larsen P, De Veylder L (2017) Modification of DNA checkpoints to confer aluminum tolerance. Trends Plant Sci **22**: 102–105

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily ofplant transcription factors. Trends Plant Sci **26**: 199–206

Ezaki B, Jayaram K, Higashi A, Takahashi K (2013) A combination of five mechanisms confers a high tolerance for aluminum to a wild species of Poaceae, *Andropogon virginicus* L. Environ Exp Bot **93**: 35–44

Fan W, Lou HQ, Yang JL, Zheng SJ (2016) The roles of STOP1-like transcription factors in aluminum and proton tolerance. Plant Signal Behav **11**: 1–4

Fernie AR, Aharoni A, Willmitzer L, Stitt M, Tohge T, Kopka J, Carroll AJ, Saito K, Fraser PD, DeLuca V (2011) Recommendations for reporting metabolite data. Plant Cell **23**: 2477–2482

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism : glycolysis , the TCA cycle and mitochondrial electron transport. Curr Opin Plant Biol **7**: 254–261

Fernie AR, Roscher A, Ratcli RG, Kruger NJ (2001) Fructose 2 , 6-bisphosphate

activates pyrophosphate : fructose-6-phos- phate 1-phosphotransferase and increases triose phosphate to hexose phosphate cycling in heterotrophic cells. 250–263

Finkemeier I, Sweetlove LJ (2009) The role of malate in plant homeostasis. F1000 Biol Rep **1**: 10–12

González L, Bolaño C, Pellissier F (2001) Use of oxygen electrode in measurements of photosynthesis and respiration. *In* RRMJ (eds), ed, Handb. Plant Ecophysiol. Tech. Springer, Dordrecht, pp 141–153

Gupta N, Gaurav SS, Kumar A (2013) Molecular basis of aluminium toxicity in plants : a review. Am J Plant Sci **4**: 21–37

Hildebrandt TM (2018) Synthesis versus degradation: directions of amino acid metabolism during Arabidopsis abiotic stress response. Plant Mol Biol **98**: 121–135

Hildebrandt TM, Nunes Nesi A, Araújo WL, Braun HP (2015) Amino acid catabolism in plants. Mol Plant **8**: 1563–1579

Hoekenga OA, Maron LG, Pineros MA, Cancado GMA, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, et al (2006) AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proc Natl Acad Sci **103**: 9738–9743

Hoekenga OA, Vision TJ, Shaff JE, Monforte AJ, Lee GP, Howell SH, Kochian LVK (2003) Identification and characterization of aluminum tolerance loci in Arabidopsis (Landsberg erecta x Columbia) by quantitative trait locus mapping. A physiologically simple but genetically complex trait. Plant Physiol **132**: 936–948

Horvath BM, Kourova H, Nagy S, Nemeth E, Magyar Z, Papdi C, Ahmad Z, Sanchez-Perez GF, Perilli S, Blilou I, et al (2017) Arabidopsis RETINOBLASTOMA RELATED directly regulates DNA damage responses through functions beyond cell cycle control. EMBO J **36**: 1261–1278

Hu Y, Dong Q, Yu D (2012) Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. Plant Sci **185**–**186**: 288–297

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF (2009) A Bacterial-Type ABC transporter is involved in aluminum tolerance in rice. Plant Cell **21**:

655–667

Igamberdiev AU, Eprintsev AT (2016) Organic acids: the pools of fixed carbon involved in redox regulation and energy balance in higher plants. Front Plant Sci **7**: 1–15

Illéš P, Schlicht M, Pavlovkin J, Lichtscheidl I, Baluška F, Ovečka M (2006) Aluminium toxicity in plants: Internalization of aluminium into cells of the transition zone in Arabidopsis root apices related to changes in plasma membrane potential, endosomal behaviour, and nitric oxide production. J Exp Bot **57**: 4201–4213

Ishizaki K, Schauer N, Larson TR, Graham IA, Fernie AR, Leaver CJ (2006) The mitochondrial electron transfer flavoprotein complex is essential for survival of Arabidopsis in extended darkness. Plant J **47**: 751–760

Iuchi S, Koyama H, Iuchi A, Kobayashi Y, Kitabayashi S, Kobayashi Y, Ikka T, Hirayama T, Shinozaki K, Kobayashi M (2007) Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. Proc Natl Acad Sci **104**: 9900–9905

Junior EMF, Cambraia J, Ribeiro C, Oliva MA, Oliveira JA, DaMatta FM (2014) The effect of Aluminum on the photosynthetic apparatus of two rice cultivars. Expl Agric **50**: 343–352

Kariya K, Sameeullah M, Sasaki T, Yamamoto Y (2017) Overexpression of the sucrose transporter gene NtSUT1 alleviates aluminum-induced inhibition of root elongation in tobacco (*Nicotiana tabacum* L.). Soil Sci Plant Nutr **63**: 45–54

Khan AA, McNeilly T, Collins JC (2000) Accumulation of amino acids, proline, and carbohydrates in response to aluminum and manganese stress in maize. J Plant Nutr **23**: 1303–1314

Kidd PS, Llugany M, Poschenrieder C, Gunsé B, Barceló J (2001) The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays* L.). J Exp Bot **52**: 1339–1352

Kobayashi Y, Hoekenga OA, Itoh H, Nakashima M, Saito S, Shaff JE, Maron LG, Pineros MA, Kochian L V., Koyama H (2007) Characterization of AtALMT1 expression in aluminum-inducible malate release and its role for rhizotoxic stress tolerance in Arabidopsis. Plant Physiol **145**: 843–852

Kobayashi Y, Ohyama Y, Kobayashi Y, Ito H, Iuchi S, Fujita M, Zhao CR,

Tanveer T, Ganesan M, Kobayashi M, et al (2014) STOP2 activates transcription of several genes for Al- and low pH-tolerance that are regulated by STOP1 in arabidopsis. Mol Plant **7**: 311–322

Kochian L V., Hoekenga OA, Piñeros MA (2004) How do crop plants tolerate acid soils: Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol **55**: 459–493

Kochian L V., Piñeros MA, Liu J, Magalhaes J V. (2015) Plant adaptation to acid soils: The molecular basis for crop aluminum resistance. Annu Rev Plant Biol **66**: 571–598

Kong X, Sun L, Zhou Y, Zhang M, Liu Y, Pan J, Li D (2011) ZmMKK4 regulates osmotic stress through reactive oxygen species scavenging in transgenic tobacco. Plant Cell Rep **30**: 2097–2104

Kopittke PM, Moore KL, Lombi E, Gianoncelli A, Ferguson BJ, Blamey FPC, Menzies NW, Nicholson TM, McKenna BA, Wang P, et al (2015) Identification of the primary lesion of toxic aluminum in plant roots. Plant Physiol **167**: 1402–1411

Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B, Bergmüller E, Dörmann P, Weckwerth W, Gibon Y, Stitt M, et al (2005) Systems biology GMD@CSB.DB : the Golm metabolome database. Bioinformatics **21**: 1635–1638

Kumar A, Bachhawat AK (2012) Pyroglutamic acid: Throwing light on a lightly studied metabolite. Curr Sci **102**: 288–297

Kumari M, Taylor GJ, Deyholos MK (2008) Transcriptomic responses to aluminum stress in roots of Arabidopsis thaliana. Mol Genet Genomics **279**: 339–357

Lam HM (1995) Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. Plant Cell **7**: 887–898

Lemire J, Mailloux R, Auger C, Whalen D, Appanna VD (2010) PPseudomonas fluorescens orchestrates a fine metabolic-balancing act to counter aluminium toxicity. Environ Microbiol **12**: 1384–1390

Li Z, Xing D (2011) Mechanistic study of mitochondria-dependent programmed cell death induced by aluminium phytotoxicity using fluorescence techniques. J Exp Bot **62**: 331–343

Liang C, Pineros MA, Tian J, Yao Z, Sun L, Liu J, Shaff J, Coluccio A, Kochian L

V., Liao H (2013) Low pH, aluminum, and phosphorus coordinately regulate malate exudation through GmALMT1 to improve soybean adaptation to acid soils. Plant Physiol **161**: 1347–1361

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry – based metabolite profiling in plants. Nat Protoc **1**: 387–396

Liu J, Luo X, Shaff J, Liang C, Jia X, Li Z, Magalhaes J, Kochian L V. (2012) A promoter-swap strategy between the AtALMT and AtMATE genes increased Arabidopsis aluminum resistance and improved carbon-use efficiency for aluminum resistance. Plant J **71**: 327–337

Liu J, Magalhaes J V., Shaff J, Kochian L V. (2009) Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. Plant J **57**: 389–399

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real- time quantitative PCR and the 2^(ΔΔCt) method. Methods **25**: 402–408

Luedemann A, Malotky L von, Erban A, Kopka J (2011) TagFinder: Preprocessing software for the fingerprinting and the profiling of gas chromatography–mass spectrometry based metabolome analyses. Plant Metabolomics Methods Mol Biol **860**: 255–286

Magalhaes J V., Liu J, Guimarães CT, Lana UGP, Alves VMC, Wang YH, Schaffert RE, Hoekenga OA, Piñeros MA, Shaff JE, et al (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat Genet **39**: 1156–1161

Maron LG, Pineros MA, Guimaraes CT, Magalhaes J V., Pleiman JK, Mao C, Shaff J, Belicuas SNJ, Kochian L V. (2010) Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. Plant J **61**: 728–740

Mattiello L, Begcy K, da Silva FR, Jorge RA, Menossi M (2014) Transcriptome analysis highlights changes in the leaves of maize plants cultivated in acidic soil containing toxic levels of Al^{3+}. Mol Biol Rep **41**: 8107–8116

Monte-Bello CC, Araujo EF, Martins MCM, Mafra V, Silva VCH, Celente V, Caldana C (2018) A flexible low cost hydroponic system for assessing plant responses to small molecules in sterile conditions. J Vizualized Exp 1–9

Moreno-Alvarado M, García-Morales S, Trejo-Téllez LI, Hidalgo-Contreras JV, Gómez-Merino FC (2017) Aluminum enhances growth and sugar concentration, alters macronutrient status and regulates the expression of NAC transcription factors in rice. Front Plant Sci **8**: 1–16

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant **15**: 473–497

Mustroph A, Zanetti ME, Jang CJH, Holtan HE, Repetti PP, Galbraith DW, Girke T, Bailey-serres J (2009) Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. Proc Natl Acad Sci **106**: 18843–18848

Negishi T, Oshima K, Hattori M, Kanai M, Mano S, Nishimura M, Yoshida K (2012) Tonoplast and plasma membrane- localized aquaporin-family transporters in blue hydrangea sepals of aluminum hyperaccumulating plantNo Title. PLoS One 7:

Nunes-Nesi A, Brito DS, Inostroza-Blancheteau C, Fernie AR, Araújo WL (2013) The complex role of mitochondrial metabolism in plant aluminum resistance. Trends Plant Sci **19**: 399–407

Nunes-Nesi A, Carrari F, Gibon Y, Sulpice R, Lytovchenko A, Fisahn J, Ratcliffe RG, Sweetlove LJ, Fernie AR (2007) Deficiency of mitochondrial fumarase activity in tomato plants impairs photosynthesis via an effect on stomatal function. Plant J **50**: 1093–1106

Pineros MA, Cancado GMA, Kochian L V. (2008) Novel properties of the wheat aluminum tolerance organic acid transporter (TaALMT1) revealed by electrophysiological characterization in xenopus oocytes: functional and structural implications. Plant Physiol **147**: 2131–2146

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophylls standarts by atomic absorption spectroscopy. Biochim Biophys Acta 384–394

Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, Prado FE (2009) Soluble sugars - Metabolism , sensing and abiotic stress: A complex network in the life of plants. Plant Signal Behav **4**: 388–393

Ruan Y-L (2014) Sucrose metabolism: Gateway to diverse carbon use and sugar signaling. Annu Rev Plant Biol **65**: 33–67

Ryan PR, Ditomaso JM, Kochian L V (1993) Aluminium toxicity in roots : an investigation of spatial sensivity and the role of the root cap. J Exp Bot **44**: 437–446

Sameeullah M, Sasaki T, Yamamoto Y (2013) Sucrose transporter NtSUT1 confers aluminum tolerance on cultured cells of tobacco (*Nicotiana tabacum* L.). Soil Sci Plant Nutr **59**: 756–770

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J **37**: 645–653

Sawaki Y, Iuchi S, Kobayashi Y, Kobayashi Y, Ikka T, Sakurai N, Fujita M, Shinozaki K, Shibata D, Kobayashi M, et al (2009) STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminum toxicities. Plant Physiol **150**: 281–294

Sharma T, Dreyer I, Kochian L, Piñeros MA (2016) The ALMT family of organic acid transporters in plants and their involvement in detoxification and nutrient security. Front Plant Sci **7**: 1–12

Shavrukov Y, Hirai Y (2016) Good and bad protons: Genetic aspects of acidity stress responses in plants. J Exp Bot **67**: 15–30

Sienkiewicz-porzucek A, Sulpice R, Osorio S, Krahnert I, Leisse A (2010) Mild reductions in mitochondrial NAD-dependent isocitrate activity result in altered nitrate assimilation and pigmentation but do not impact growth. Mol Plant **3**: 156–173

Silva S, Pinto G, Dias MC, Correia CM, Moutinho-Pereira J, Pinto-Carnide O, Santos C (2012) Aluminium long-term stress differently affects photosynthesis in rye genotypes. Plant Physiol Biochem **54**: 105–112

Singh R, Lemire J, Mailloux RJ, Chénier D, Hamel R, Appanna VD (2009) An ATP and oxalate generating variant tricarboxylic acid cycle counters aluminum toxicity in *Pseudomonas fluorescens*. PLoS One 4:

Sivaguru M, Horst WJ (1998) The distal part of the transition zone is the most aluminum-sensitive apical root zone of maize. Plant Physiol **116**: 155–163

Sulpice R, Pyl E, Ishihara H, Trenkamp S, Steinfath M, Witucka-wall H, Korff M

Von, Caroline M, Gibon Y, Stitt M (2009) Starch as a major integrator in the regulation of plant growth. Proc Natl Acad Sci **106**: 10348–10353

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG (2010) Not just a circle: Flux modes in the plant TCA cycle. Trends Plant Sci **15**: 462–470

Toda T, Koyama H, Hori T, Hara T (1999) Aluminum tolerance of *Arabidopsis thaliana* under hydroponic and soil culture conditions. Soil Sci Plant Nutr **45**: 419–425

Truffault V, Fry SC, Stevens RG, Gautier H (2017) Ascorbate degradation in tomato leads to accumulation of oxalate, threonate and oxalyl threonate. Plant J **89**: 996–1008

Uexkull HR von, Mutert E (1995) Global extent , development and economic impact of acid soils. Plant Soil **171**: 1–15

Xia J, Yamaji N, Kasai T, Ma JF (2010) Plasma membrane-localized transporter for aluminum in rice. Proc Natl Acad Sci **107**: 18381–18385

Yang L, Tian D, Todd CD, Luo Y, Hu X (2013) Comparative proteome analyses reveal that nitric oxide is an important signal molecule in the response of rice to aluminum toxicity. J Proteome Res **12**: 1316–1330

Ying X, Hui Z, Ying J, Fan W, Wu H, Yuan M (2013) Association of specific pectin methylesterases with Al-induced root elongation inhibition in rice. 502–511

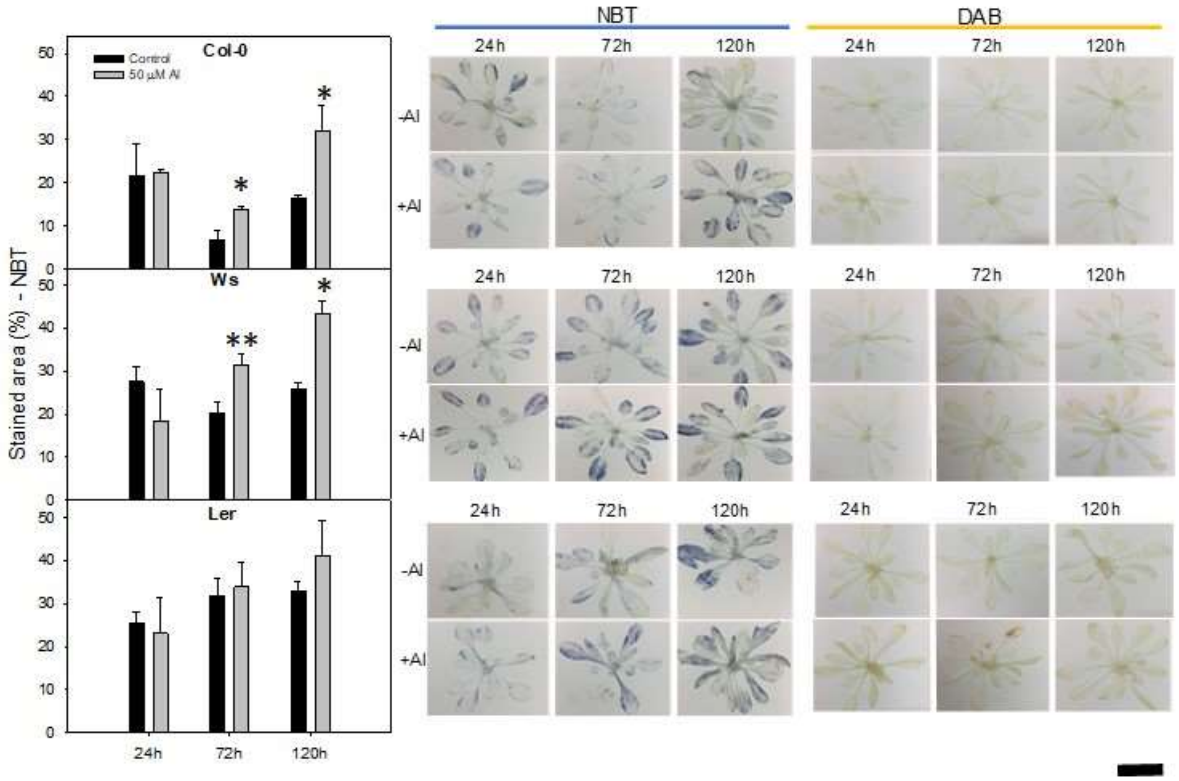
Yokosho K, Yamaji N, Ma JF (2011) An Al-inducible MATE gene is involved in external detoxification of Al in rice. Plant J **68**: 1061–1069

Zhu XF, Shi YZ, Lei GJ, Fry SC, Zhang BC, Zhou YH, Braam J, Jiang T, Xu XY, Mao CZ, et al (2012) XTH31, Encoding an in vitro XEH/XET-Active enzyme, regulates aluminum a sensitivity by modulating in vivo XET action, cell wall xyloglucan content, and aluminum binding capacity in Arabidopsis. Plant Cell **24**: 4731–4747

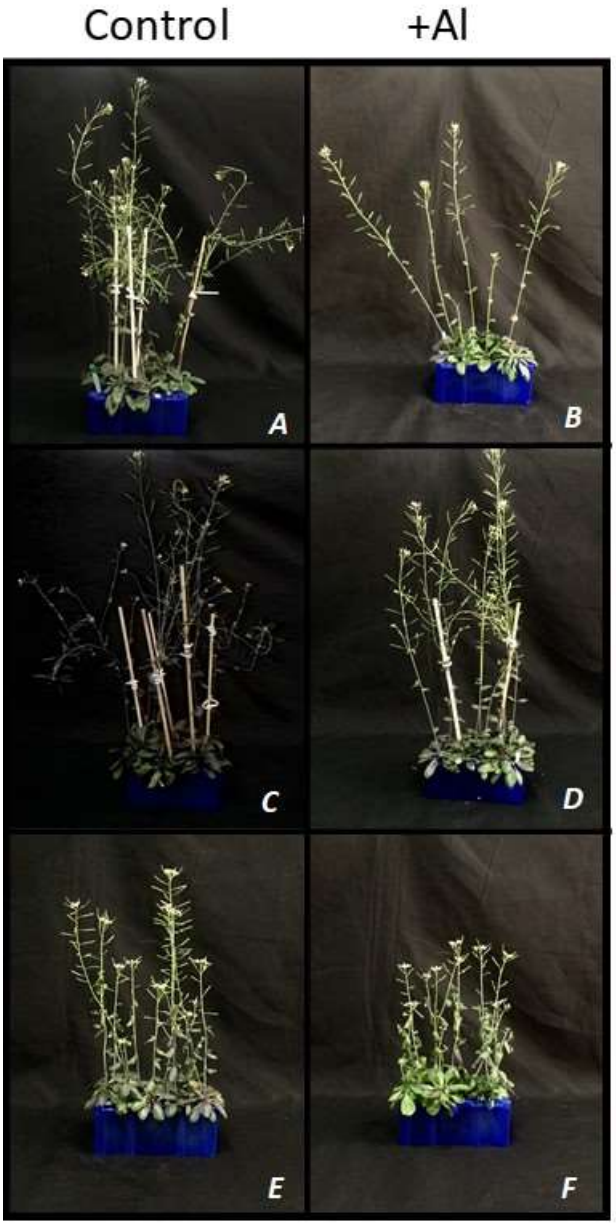
Zhu XF, Wan JX, Sun Y, Shi YZ, Braam J, Li GX, Zheng SJ (2014) Xyloglucan Endotransglucosylase-Hydrolase17 interacts with Xyloglucan Endotransglucosylase-Hydrolase31 to Confer Xyloglucan Endotransglucosylase action and affect aluminum sensitivity in Arabidopsis. Plant Physiol **165**: 1566–1574

7. SUPPLEMENTAL MATERIAL

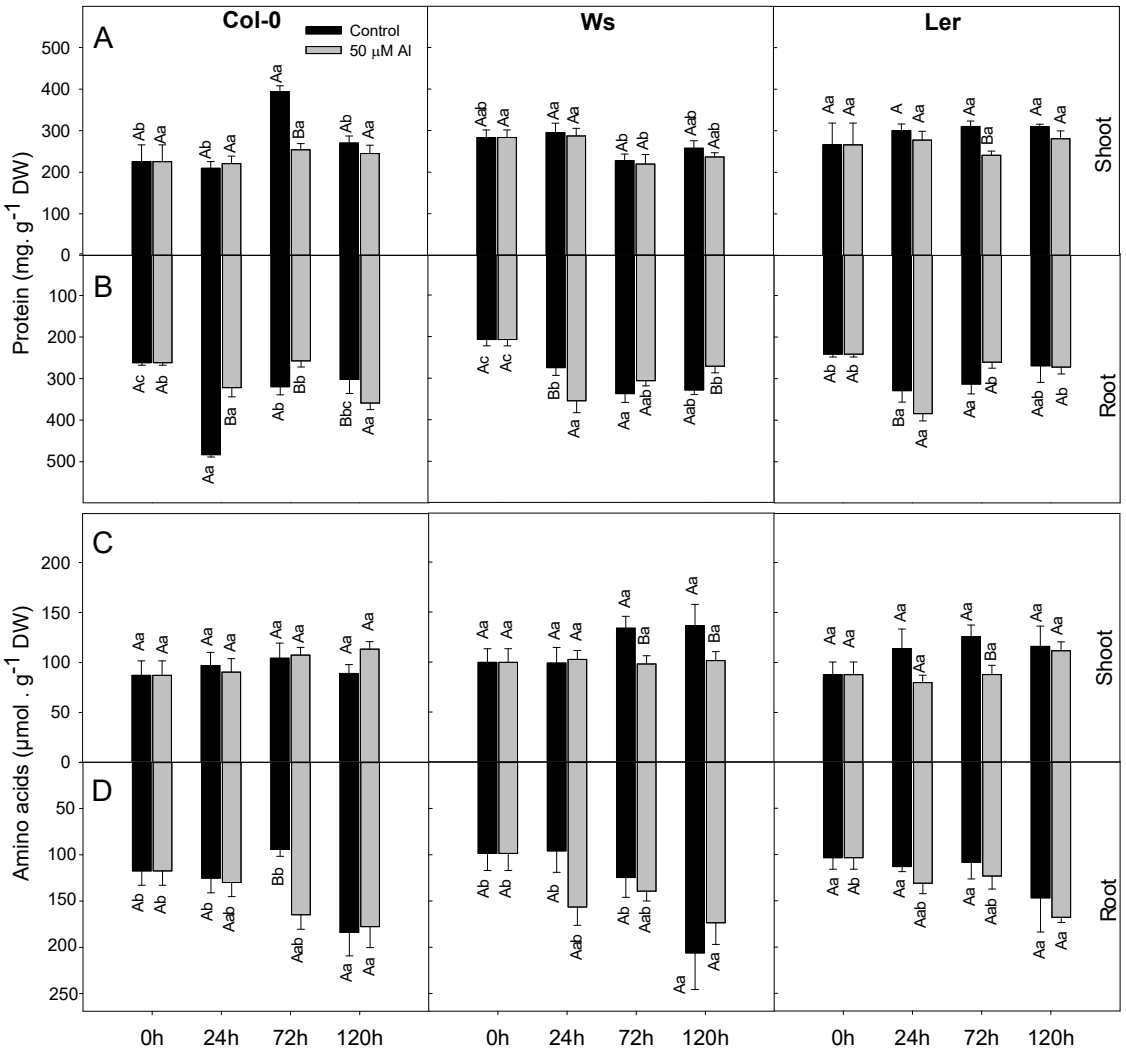
7.1. Supplemental Figures



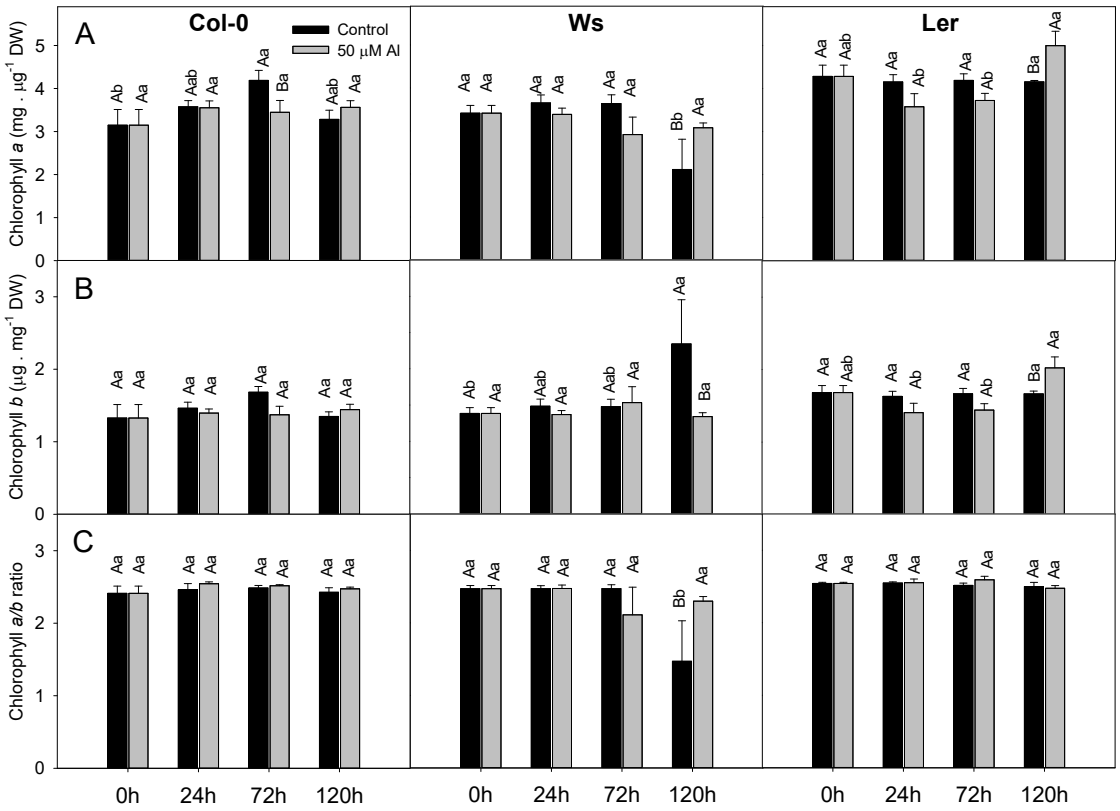
Supplemental figure S1: Reactive oxygen species histochemical assay of Arabidopsis ecotypes' shoot of Al-stressed plants. Percentage (%) of stained area with Nitroblue tetrazolium (NBT) reagent (graph) in control (-Al) and Al-stressed (+Al) plants. Pictures of stained O_2^- (blue bar - NBT) and H_2O_2 (yellow bar - DAB) of plant exposed to control and Al stress for three days. Treated plants were compared with the rspective control at harvesting time. Values are presented as means ± SE (n=3). One asterisk indicate that the percentage values of stained area in treated plants were significantly different ($P < 0.05$) when compared with the respective controls. Two asterisks indicate significantly different values with $P < 0.10$. Black bar = 1.5 cm



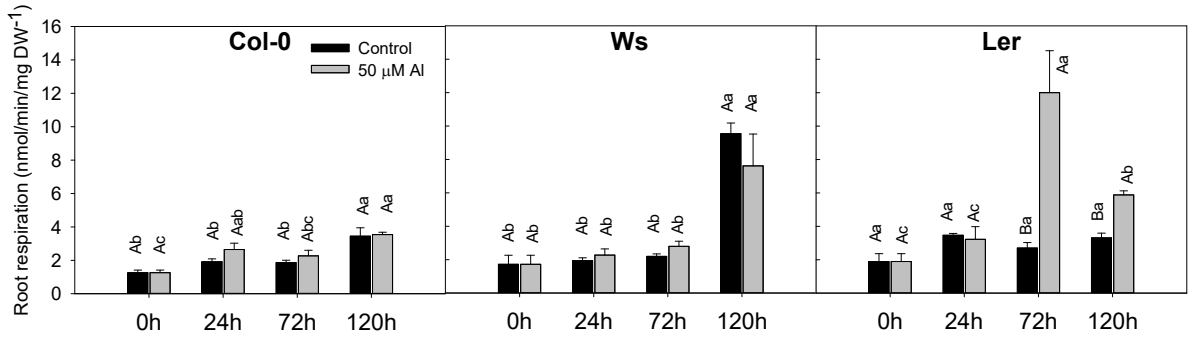
Supplemental figure S2: Flowering stage Arabidopsis phenotype imposed to long-term Al stress. Plants were kept under control (pH 4.5) hydroponic environment and with higher Al stress conditions (100 µM Al^{3+}) until the end of reproductive stage to yield components evaluations (*i.e.* number of siliques, siliques length, number of seeds and seed weight). A, Col-0 control; B, Col-0 + Al; C, Ws control; D, Ws + Al; E, Ler control; F, Ler + Al.



Supplemental figure S3: Shoot and root protein and amino acids content in Arabidopsis ecotypes under Al stress. Protein (Shoot – A; Roots - B) and amino acid (Shoot – C; Roots - D) content in the ecotypes evaluated in both tissues (*i.e.* leaves and roots) for five days Al stress. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.



Supplemental figure S4: Chlorophylls *a* (A) and *b* (B) content of the three studied ecotypes and the respective *a/b* ratio (C) under five days Al stress. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different (P < 0.05, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.



Supplemental figure S5: Root respiration rate in Arabidopsis ecotypes evaluated during Al stress. The measurement was carried out with Clark electrode. Values presented are means of ± SE of four biological replicates, with two whole roots each; Different letters represent average values that were judged to be statistically different between genotypes (P < 0.05, Tukey test). Capital letters represent statistical differences within control (pH 4.5) and Al environment (50 µM Al), and lower-case letters demonstrate statistical differences along harvesting times.

7.2. Supplemental Tables

Supplemental tableS1: Primers utilized to qRT-PCR.

Gene	Locus	Sequence
ACT	AT2G37620	Fwd 5'-CTTGCACCAAGCA GCATGAA-3'
		Rev 5'-CCGATCCAGACACTGTACTTCCTT-3'
ALMT1	AT1G08430	Fwd 5'-GGTCATGTTGGTCTTTGTGCAAGC-3'
		Rev 5'-CGACAACGATATCAGCGCGAAC-3'
WRKY46	AT2G46400	Fwd 5'-ACCTGCTGCTGTTGAGAATTCCG-3'
		Rev 5'-ACGACCACAACCAATCCTGTCC-3'
STOP1	AT1G34370	Fwd 5'- TCTGCACGATATGTGGCAAGGG-3'
		Rev5'-CATCTCCATGCCCTCTCATATGC-3'
MATE1	AT1G51340.1	Fwd 5'-TCACTTCTTGCAGACGGGTACG-3'
		Rev 5'-AACCGAGAACCAATCCCAACTGC-3'
SUC2	AT1G22710	Fwd 5'-TAGCCATTGTCGTCCCTCAGATG-3'
		Rev 5'-ACCACCGAATAGTTCGTCGAATGG-3'
GST1	AT1G02930	Fwd 5'-GAGCCTTTCATCCTTCGCAACC-3'
		Rev 5'-CATGTCCTTGCCAGTTGAGAGAAG-3'
ZAT12	AT5G59820	Fwd 5'-TGCGAGTCACAAGAAGCCTA-3'
		Rev 5'- GTGTCCTCCCAAAGCTTGTC-3'

ALMT1, Aluminum Activated Malate Transporter 1; *WRKY 46*; *STOP 1*, Sensitive To Proton Rhyzotoxicity 1; *MATE 1*, Multidrug and Toxic Compound Extrusion 1; *SUC* 2, Sucrose-proton Symporter 2; *GST 1*, Glutathione S-transferase 1; *ZAT 12*.

Supplemental table S2: Biometric data from the five days hidroponic experiment (vegetative stage) and from plants kept under hidroponic environment to yield evaluations (reproductive stage). Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within the treatments, and lower case letters demonstrate statistical differences between ecotypes.

	Vegetative stage	
	pH 4.5	**50 µM Al**
Final shoot biomass (mg DW)		
Col-0	8.73 ± 0.69Aa	6.12 ± 0.55Ba
Ws	8.79 ± 0.36Aa	7.03 ± 0.45Ba
Ler	6.91 ± 0.64Ab	6.82 ± 0.18Aa
Final root biomass (mg DW)		
Col-0	1.51 ± 0.06Aa	1.49 ± 0.16Aa
Ws	1.91 ± 0.26Aa	1.70 ± 0.04Aa
Ler	1.57 ± 0.18Aa	1.63 ± 0.12Aa
Root / Shoot ratio		
Col-0	0.18 ± 0.01Ba	0.26 ± 0.04Aa
Ws	0.22 ± 0.03Aa	0.24 ± 0.01Aa
Ler	0.24 ± 0.02Aa	0.24 ± 0.02Aa
	Reproductive stage (yield)	
	pH 4.5	**100 µM Al**
Silique number (units / plant)		
Col-0	128.90 ± 7.77Ab	67.51 ± 8.84Ba
Ws	156.80 ± 10.56Aa	89.35 ± 2.79Ba
Ler	98.88 ± 5.95Ac	69.00 ± 3.72Ba
Siliques length (mm)		
Col-0	12.09 ± 0.10Aab	12.33 ± 0.25Aa
Ws	12.48 ± 0.07Aa	12.12 ± 0.08Aab
Ler	11.62 ± 0.26Ab	11.44 ± 0.44Ab
Thousand seeds weight (mg)		
Col-0	19.08 ± 0.07Aa	18.73 ± 0.21Aa
Ws	19.39 ± 0.20Aa	18.07 ± 0.07Bb
Ler	17.93 ± 0.13Ab	18.21 ± 0.16Aab
Number of seeds/silique		
Col-0	44.71 ± 0.51Ab	46.73 ± 0.70Aa
Ws	47.10 ± 0.60Aab	44.55 ± 0.60Ba
Ler	48.00 ± 0.86Aa	46.60 ± 0.95Aa

Supplemental table S3: **Germination and root growth rates of three Arabidopsis ecotypes grown in agar plates containing Zhu nutrient solution and four different conditions, being pH 5.7 (pH control), pH 4.5 (Al control), 25 µM and 100 µM Al**. The growth conditions were carried out as detailed in M&M. For germination, the evaluations were carried out every 6 hours after 24h of light exposure, and after the third counting point evaluations began to be made every 24 hours until the fifth day. For root growth rate, the evaluations were made every two days after most of the seeds germinated (2 days after light exposure). Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within the treatments, and lower-case letters demonstrate statistical differences along days.

	Germination rate (%)											
Col-0	**24h**		**30h**		**36h**		**48h**		**72h**		**96h**		**120h**	
pH 5.7	54.00 ± 9.63	Abb	92.67 ± 1.25	A	94.67 ± 0.82	Aa	96.00 ± 0.67	Aa	98.00 ± 0.82	Aa	98.00 ± 0.82	Aa	98.00 ± 0.82	Aa
pH 4.5	57.33 ± 5.72	ABb	90.00 ± 1.49	Aa	94.00 ± 1.25	Aa	96.00 ± 1.63	Aa	98.00 ± 0.82	Aa	98.00 ± 0.82	Aa	98.00 ± 0.82	Aa
25 µM	64.00 ± 5.31	Ab	93.33 ± 1.83	Aa	96.00 ± 2.67	Aa	96.67 ± 2.11	Aa	98.67 ± 0.82	Aa	98.67 ± 0.82	Aa	98.67 ± 0.82	Aa
100 µM	50.83 ± 6.58	Bc	84.17 ± 1.60	Ab	90.00 ± 3.60	Aab	92.50 ± 2.85	Aab	96.67 ± 1.36	Aa	96.67 ± 1.36	Aa	96.67 ± 1.36	Aa
Ws														
pH 5.7	34.00 ± 9.27	Bb	94.67 ± 2.71	Aa	98.00 ± 0.82	Aa	99.33 ± 0.67	Aa	100.00 ± 0.00	Aa	100.00 ± 0.00	Aa	100.00 ± 0.00	Aa
pH 4.5	51.67 ± 11.34	Ab	90.83 ± 2.85	Aa	95.00 ± 0.96	Aa	96.67 ± 0.00	Aa	97.50 ± 0.83	Aa	97.50 ± 0.83	Aa	97.50 ± 0.83	Aa
25 µM	32.00 ± 5.33	Bb	88.67 ± 2.49	Aa	94.67 ± 1.70	Aa	96.00 ± 1.25	Aa	98.67 ± 0.82	Aa	98.67 ± 0.82	Aa	98.67 ± 0.82	Aa
100 µM	31.67 ± 4.81	Bb	88.33 ± 2.15	Aa	95.00 ± 1.67	Aa	97.50 ± 1.60	Aa	98.33 ± 1.67	Aa	98.33 ± 1.67	Aa	98.33 ± 1.67	Aa
Ler														
pH 5.7	4.67 ± 2.26	Bc	66.00 ± 7.02	Bb	83.33 ± 4.47	Aa	88.00 ± 3.43	Aa	92.00 ± 2.71	Aa	92.00 ± 2.71	Aa	92.00 ± 2.71	Ac
pH 4.5	8.33 ± 3.97	Ac	64.17 ± 4.38	Ab	80.00 ± 3.33	Aab	85.00 ± 3.97	Aa	92.50 ± 2.10	Aa	92.50 ± 2.10	Aa	92.50 ± 2.10	Aa
25 µM	0.67 ± 0.67	Bd	58.67 ± 3.27	BCc	80.00 ± 2.98	Ab	87.33 ± 2.45	Aab	94.67 ± 0.82	Aa	94.67 ± 0.82	Aa	94.67 ± 0.82	Aa
100 µM	0.00 ± 0.00	Bd	54.17 ± 6.71	Cc	75.00 ± 3.75	Ab	84.17 ± 4.28	Aab	90.83 ± 1.43	Aa	90.83 ± 1.43	Aa	90.83 ± 1.43	Aa

Supplemental Table S3 extension

Root growth rate (mm)												
Col-0	**2 days**	**4 days**	**6 days**	**8 days**	**10 days**	**12 days**	**14 days**
pH 5.7	1.74 ± 0.38 Ag	4.39 ± 0.34 Af	7.11 ± 0.44 Ae	9.06 ± 0.61 Ad	10.96 ± 0.62 Ac	13.03 ± 0.76 Ab	15.99 ± 1.03 Aa
pH 4.5	1.60 ± 0.24 Abf	3.56 ± 0.30 Abe	5.17 ± 0.36 Bde	6.59 ± 0.37 Bcd	7.71 ± 0.46 Bbc	8.65 ± 0.60 Bab	9.93 ± 0.91 Ba
25 µM	0.75 ± 0.19 Abe	2.53 ± 0.21 BCd	3.92 ± 0.30 BCcd	4.84 ± 0.27 Cbc	5.52 ± 0.32 Cab	6.39 ± 0.35 Cab	7.29 ± 0.39 Ca
100 µM	0.10 ± 0.05 Bd	1.17 ± 0.18 Ccd	2.41 ± 0.17 Cbc	3.01 ± 0.14 Dab	3.59 ± 0.17 Dab	4.11 ± 0.17 Dab	4.60 ± 0.17 Da
Ws							
pH 5.7	1.41 ± 0.35 Af	3.71 ± 0.55 Ae	6.17 ± 0.76 Ad	8.44 ± 0.81 Acd	10.60 ± 0.88 Ac	13.79 ± 1.13 Ab	18.37 ± 1.95 Aa
pH 4.5	1.42 ± 0.15 Af	3.59 ± 0.21 Aef	5.38 ± 0.22 Ade	7.01 ± 0.29 Abcd	8.52 ± 0.35 Bbc	10.38 ± 0.64 Bab	12.51 ± 0.97 Ba
25 µM	1.16 ± 0.15 Ae	3.10 ± 0.08 Abde	4.71 ± 0.14 Acd	5.74 ± 0.13 Bbc	6.34 ± 0.29 Babc	7.55 ± 0.42 Cab	8.62 ± 0.69 Ca
100 µM	0.16 ± 0.10 Ac	1.20 ± 0.15 Bbc	2.33 ± 0.22 Babc	2.95 ± 0.15 Cab	3.42 ± 0.17 Cab	3.88 ± 0.15 Da	4.33 ± 0.17 Da
Ler							
pH 5.7	1.15 ± 0.33 Af	4.04 ± 0.56 Ae	6.41 ± 0.48 Ad	7.60 ± 0.49 Acd	9.50 ± 0.63 Abc	11.11 ± 0.86 Ab	12.89 ± 1.31 Aa
pH 4.5	1.28 ± 0.28 Ae	3.40 ± 0.33 Ad	5.04 ± 0.32 Abcd	5.91 ± 0.35 Bbc	6.95 ± 0.36 Bb	7.81 ± 0.32 Bab	8.68 ± 0.39 Ba
25 µM	0.82 ± 0.08 Ae	2.68 ± 0.12 Abd	4.30 ± 0.13 Bcd	5.22 ± 0.26 Bbc	5.96 ± 0.44 Bab	6.85 ± 0.34 Bab	7.45 ± 0.40 Ba
100 µM	0.19 ± 0.10 Ae	1.39 ± 0.16 Bde	2.68 ± 0.22 Ccd	3.54 ± 0.27 Cbc	4.25 ± 0.25 Cabc	4.83 ± 0.24 Cab	5.46 ± 0.34 Ca

Supplemental tableS4: Shoot biomass and final root length in response to Al stress of three Arabidopsis ecotypes grown in agar plates environment. The agar plates containing Zhu nutrient solution and four different conditions, being pH 5.7 (pH control), pH 4.5 (Al control), 25 µM and 100 µM Al^{3+}.The growth conditions were carried out as detailed in M&M. Values are presented as means ± SE (n=5). Different letters represent average values that were judged to be statistically different ($P < 0.05$, Tukey test). Capital letters represent statistical differences within the treatments, and lower-case letters demonstrate statistical differences between ecotypes.

	pH 5.7	**pH 4.5**	**25 µM**	**100 µM**
Shoot biomass (mg DW. plant^{-1})				
Col-0	0.49 ± 0.04 Aab	0.30 ± 0.02 Ba	0.49 ± 0.03 Ab	0.28 ± 0.01 Ba
Ws	0.58 ± 0.04 Aa	0.30 ± 0.02 Ba	0.63 ± 0.05 Aa	0.29 ± 0.01 Ba
Ler	0.44 ± 0.04 Ab	0.24 ± 0.01 Ba	0.46 ± 0.05 Ab	0.27 ± 0.03 Ba
Final Shoot Length (mm)				
Col-0	15.99 ± 0.89 Aa	9.93 ± 0.91 Bab	7.29 ± 0.39 BCa	4.60 ± 0.17 Ca
Ws	18.37 ± 1.69 Aa	12.51 ± 0.97 Ba	8.62 ± 0.69 Ca	4.33 ± 0.17 Da
Ler	12.89 ± 1.14 Ab	8.68 ± 0.39 Bb	7.45 ± 0.40 BCa	5.46 ± 0.34 Ca

Supplemental tableS5 - Shoot Relative GC-MS metabolite content in the *A. thaliana* ecotypes under AI treatment (4-week-old). Values are presented by means ± SE (n=4). Bold numbers represent values that were significantly different ($P < 0.05$) when comparing controls with timepoint 0h and treatment values with their respective control. Both determined by Student's *t* test.

	Shoot	Col-0													
	Metabolite	0h	error	24h	error	24hT	error	72h	error	72hT	error	120h	error	120hT	error
Amino acids	β - Alanine	3.25	0.81	**1.17**	**0.13**	1.50	0.24	2.02	0.23	1.36	0.18	1.82	0.16	1.36	0.37
	Asparagine	0.63	0.14	0.55	0.11	0.91	0.11	0.67	0.08	**2.43**	**0.58**	1.00	0.15	**2.45**	**0.36**
	Aspartate	4.01	0.26	3.98	0.62	4.80	0.44	**2.09**	**0.19**	**5.39**	**0.31**	**2.83**	**0.33**	**4.69**	**0.33**
	Cysteine	0.67	0.14	0.61	0.14	0.76	0.06	0.72	0.07	**1.25**	**0.22**	1.05	0.12	1.44	0.24
	Glutamate	8.08	1.18	7.57	0.63	**11.31**	**0.69**	7.85	0.25	**12.75**	**1.36**	9.09	0.70	10.37	0.69
	Glutamine	7.16	1.66	5.26	1.46	8.11	1.34	7.06	0.76	**29.25**	**8.44**	8.02	1.83	**34.42**	**4.56**
	Glycine	6.42	1.67	10.72	0.99	15.43	3.24	**12.35**	**1.41**	11.67	2.33	**15.93**	**2.23**	10.94	0.41
	Ornithine	0.31	0.06	0.22	0.05	0.40	0.07	0.26	0.03	**0.60**	**0.08**	0.46	0.06	**1.27**	**0.17**
	Phenylalanine	0.48	0.07	0.42	0.06	0.57	0.07	0.41	0.01	0.40	0.02	0.38	0.03	0.44	0.05
	Proline	25.17	0.16	**11.87**	**2.03**	14.36	1.08	**10.07**	**0.32**	**18.11**	**1.84**	**15.41**	**2.92**	**25.84**	**1.51**
	Serine	17.99	1.52	**10.94**	**1.64**	14.07	0.73	**13.21**	**0.15**	19.24	3.49	16.68	0.88	19.00	0.86
	Threonine	6.79	0.42	**4.78**	**0.29**	5.43	0.24	7.08	0.32	**4.66**	**0.24**	6.57	0.18	6.17	0.64
	Valine	2.85	0.15	2.07	0.30	2.01	0.11	**2.40**	**0.09**	**1.75**	**0.10**	2.55	0.24	2.12	0.29
Sugars and Sugars alcohol	Erythritol	0.28	0.06	0.22	0.04	0.21	0.01	**0.15**	**0.01**	0.17	0.01	0.15	0.02	0.14	0.01
	Fructose	118.49	44.53	53.88	17.91	80.32	10.75	29.00	5.73	**198.97**	**6.66**	45.26	5.76	**143.39**	**30.49**
	Glucose	184.10	60.15	139.50	31.95	199.23	22.25	78.64	25.96	**198.75**	**2.46**	94.50	16.70	**190.38**	**19.88**
	Myo-inositol	17.10	1.55	25.93	6.23	31.82	1.26	**9.22**	**1.08**	**34.90**	**4.13**	**8.79**	**1.76**	**29.31**	**4.44**
	Sucrose	102.42	16.25	71.24	5.00	**89.27**	**5.48**	85.06	5.78	94.89	6.48	70.78	9.36	82.08	7.17
	Trehalose	0.40	0.05	0.58	0.08	0.56	0.03	**0.59**	**0.02**	**0.93**	**0.05**	**0.67**	**0.08**	**1.16**	**0.05**
	Galactinol	1.94	0.82	4.68	2.03	5.29	1.27	0.63	0.05	**4.85**	**0.79**	0.39	0.13	**5.61**	**0.65**
	Raffinose	2.02	0.81	4.03	1.67	3.97	0.85	0.38	0.08	**3.30**	**0.46**	0.45	0.16	**4.98**	**0.84**
Organic acids	Glycerate	1.67	0.28	2.57	0.50	1.74	0.08	1.36	0.10	**3.06**	**0.20**	1.41	0.06	**2.61**	**0.08**
	Succinate	2.01	0.35	3.52	0.80	3.64	0.11	1.82	0.06	**5.22**	**0.57**	2.08	0.33	3.08	0.53
	Fumarate	223.98	64.28	407.23	74.98	414.99	22.71	137.05	28.81	**432.66**	**22.83**	113.22	31.83	**258.97**	**38.79**
	Malate	4.68	0.85	7.68	1.69	7.45	1.09	**2.29**	**0.31**	**8.12**	**0.35**	**2.01**	**0.24**	**5.76**	**1.08**
	GABA	2.78	0.77	1.41	0.08	**1.17**	**0.05**	1.41	0.14	1.66	0.08	1.40	0.14	**2.07**	**0.16**
	Threonate	6.16	0.31	**12.66**	**1.49**	11.79	0.23	**12.00**	**1.01**	**20.76**	**1.22**	**10.31**	**1.54**	**17.69**	**0.50**
	Pyroglutamate	81.18	12.14	53.95	4.29	**105.71**	**8.30**	54.76	4.27	**131.70**	**16.50**	76.04	12.41	**147.69**	**15.10**
	Citrate	3.20	0.31	3.87	0.78	3.03	0.21	**0.96**	**0.09**	**2.44**	**0.10**	**0.96**	**0.12**	1.25	0.23
Others	Glycerol	8.50	0.96	6.17	0.40	5.97	0.26	6.29	0.24	6.38	0.17	**5.06**	**0.29**	**6.30**	**0.20**
	Putrescine	1.20	0.43	1.28	0.32	1.58	0.32	1.53	0.11	**1.13**	**0.11**	1.85	0.25	2.10	0.49
	Dehydroascorbate	0.32	0.07	0.54	0.10	**0.24**	**0.02**	0.39	0.07	0.33	0.01	0.18	0.03	0.20	0.03
	Spermidine	0.11	0.01	0.16	0.03	0.11	0.00	0.18	0.03	**0.09**	**0.01**	**0.21**	**0.03**	0.17	0.03

	Shoot	Ws															
	Metabolite	0h	error	24h	error	24hT	error	72h	error	72hT	error	120h	error	120hT	error		
Amino acids	β - Alanine	1.49	± 0.46	1.34	± 0.18	1.18	± 0.13	2.76	± 0.45	1.96	± 0.12	1.16	± 0.02	1.02	± 0.10		
	Asparagine	0.88	± 0.29	1.01	± 0.24	2.00	± 0.42	1.09	± 0.22	1.93	± 0.65	1.30	± 0.14	**2.36**	**± 0.39**		
	Aspartate	2.79	± 0.81	4.22	± 0.96	3.67	± 0.20	4.66	± 0.77	5.24	± 0.39	2.07	± 0.19	**3.46**	**± 0.41**		
	Cysteine	0.75	± 0.22	0.65	± 0.11	1.18	± 0.20	0.91	± 0.08	**1.40**	**± 0.18**	0.64	± 0.03	**1.21**	**± 0.20**		
	Glutamate	6.65	± 1.74	7.38	± 0.48	8.21	± 0.82	8.86	± 0.62	12.35	± 1.51	8.69	± 0.39	10.86	± 1.36		
	Glutamine	6.25	± 2.00	5.47	± 1.52	**17.64**	**± 2.92**	**16.12**	**± 1.10**	**35.39**	**± 8.06**	10.94	± 1.02	**26.84**	**± 4.84**		
	Glycine	6.04	± 0.31	**11.28**	**± 1.42**	**27.79**	**± 0.77**	**12.39**	**± 0.97**	13.68	± 0.78	**13.09**	**± 1.93**	13.29	± 1.81		
	Ornithine	0.19	± 0.07	0.27	± 0.07	**0.52**	**± 0.06**	**0.62**	**± 0.11**	**1.29**	**± 0.12**	0.89	± 0.38	1.56	± 0.29		
	Phenylalanine	0.65	± 0.03	0.53	± 0.05	**0.75**	**± 0.07**	0.55	± 0.08	0.52	± 0.05	**0.41**	**± 0.01**	**0.52**	**± 0.04**		
	Proline	9.38	± 2.10	**145.52**	**± 33.18**	**41.64**	**± 6.00**	**29.69**	**± 6.76**	22.39	± 4.30	38.55	± 17.65	31.85	± 9.33		
	Serine	18.87	± 1.92	13.34	± 1.25	**22.01**	**± 0.84**	17.14	± 1.81	20.72	± 1.53	**14.27**	**± 0.57**	17.97	± 2.21		
	Threonine	6.45	± 0.66	5.03	± 0.23	6.28	± 0.51	8.11	± 0.93	5.71	± 0.35	5.86	± 0.39	**7.08**	**± 0.21**		
	Valine	3.71	± 0.30	**2.19**	**± 0.15**	**2.84**	**± 0.05**	3.26	± 0.39	2.55	± 0.13	**2.04**	**± 0.03**	2.33	± 0.10		
Sugars and Sugars alcohol	Erythritol	0.23	± 0.08	0.18	± 0.03	0.20	± 0.01	0.17	± 0.02	0.13	± 0.01	0.16	± 0.02	0.11	± 0.01		
	Fructose	74.43	± 26.79	102.73	± 11.98	152.30	± 20.71	34.52	± 11.11	**162.77**	**± 9.99**	87.62	± 19.88	94.56	± 18.11		
	Glucose	174.87	± 69.17	164.15	± 3.14	**214.62**	**± 11.49**	110.01	± 36.27	178.27	± 9.67	123.26	± 18.78	131.60	± 20.59		
	Myo-inositol	23.66	± 8.63	23.74	± 4.46	30.35	± 2.29	16.22	± 1.59	**37.13**	**± 4.39**	13.80	± 2.00	20.89	± 3.28		
	Sucrose	64.69	± 20.22	71.92	± 11.25	69.60	± 1.17	98.64	± 5.88	97.57	± 10.21	85.34	± 3.29	85.35	± 7.62		
	Trehalose	0.52	± 0.18	0.59	± 0.03	0.70	± 0.08	0.87	± 0.13	**1.32**	**± 0.08**	0.69	± 0.10	**0.97**	**± 0.04**		
	Galactinol	4.68	± 2.09	3.52	± 1.19	3.13	± 0.50	2.40	± 0.61	5.16	± 1.41	2.15	± 1.05	2.22	± 0.64		
	Raffinose	5.15	± 2.47	2.25	± 0.76	2.16	± 0.45	2.32	± 0.42	4.40	± 1.50	0.81	± 0.22	1.77	± 0.49		
Organic acids	Glycerate	1.29	± 0.38	1.86	± 0.32	1.40	± 0.06	1.65	± 0.02	**2.55**	**± 0.20**	1.46	± 0.03	**2.55**	**± 0.21**		
	Succinate	1.69	± 0.55	3.35	± 0.60	4.16	± 0.49	2.28	± 0.05	**4.60**	**± 0.34**	2.59	± 0.30	2.32	± 0.24		
	Fumarate	229.53	± 76.04	390.19	± 45.50	298.35	± 15.10	221.52	± 60.31	304.50	± 8.17	204.92	± 34.21	192.86	± 23.72		
	Malate	2.57	± 0.90	4.95	± 1.20	4.44	± 0.40	4.73	± 0.70	6.45	± 0.35	3.10	± 0.09	**4.33**	**± 0.18**		
	GABA	1.69	± 0.65	1.75	± 0.37	1.74	± 0.23	1.83	± 0.15	2.21	± 0.08	1.36	± 0.07	1.75	± 0.26		
	Threonate	6.45	± 1.90	11.73	± 0.75	14.43	± 0.89	**15.15**	**± 1.03**	**19.07**	**± 0.77**	9.39	± 0.24	**15.94**	**± 0.62**		
	Pyroglutamate	91.25	± 17.10	71.65	± 8.97	**146.00**	**± 6.21**	94.14	± 7.68	**165.16**	**± 11.72**	73.54	± 10.60	121.73	± 18.20		
	Citrate	1.90	± 0.76	2.92	± 1.13	2.52	± 0.65	2.31	± 0.44	1.63	± 0.16	1.00	± 0.04	1.29	± 0.16		
Others	Glycerol	12.00	± 2.06	7.52	± 1.46	6.40	± 0.35	**7.02**	**± 0.54**	6.59	± 0.18	**5.36**	**± 0.35**	6.43	± 0.87		
	Putrescine	1.69	± 0.27	1.61	± 0.35	**3.13**	**± 0.28**	1.99	± 0.45	2.19	± 0.35	1.82	± 0.25	**2.68**	**± 0.05**		
	Dehydroascorbate	0.17	± 0.05	0.20	± 0.03	0.18	± 0.03	0.23	± 0.03	0.20	± 0.04	0.14	± 0.01	0.17	± 0.01		
	Spermidine	0.16	± 0.05	0.14	± 0.02	0.13	± 0.01	0.12	± 0.02	0.11	± 0.01	0.20	± 0.04	0.17	± 0.03		

	Shoot	Ler																			
	Metabolite	0h		error		24h		error		24hT		error		72h		error		72hT		error		120h		error		120hT		error
Amino acids	β - Alanine	1.67	±	0.39		**2.67**	**±**	**0.10**		**1.13**	**±**	**0.29**		1.91	±	0.21		1.76	±	0.31		3.20	±	0.65		6.03	±	1.95
	Asparagine	0.85	±	0.20		1.13	±	0.08		1.44	±	0.42		1.06	±	0.09		1.95	±	0.49		1.03	±	0.10		1.15	±	0.22
	Aspartate	5.53	±	0.32		**3.89**	**±**	**0.44**		4.67	±	0.34		4.49	±	0.75		5.82	±	0.12		**3.26**	**±**	**0.20**		**6.48**	**±**	**1.01**
	Cysteine	0.55	±	0.02		**0.72**	**±**	**0.06**		0.63	±	0.12		0.71	±	0.11		**1.27**	**±**	**0.08**		**0.91**	**±**	**0.10**		1.15	±	0.20
	Glutamate	6.89	±	0.87		**10.00**	**±**	**0.52**		13.92	±	2.89		10.40	±	2.00		11.83	±	1.37		**9.78**	**±**	**0.38**		8.36	±	0.79
	Glutamine	11.99	±	3.11		15.08	±	1.82		24.26	±	8.14		14.00	±	0.75		23.33	±	3.70		9.70	±	0.92		**14.42**	**±**	**1.59**
	Glycine	9.93	±	2.23		8.26	±	1.87		5.13	±	0.53		8.40	±	1.48		6.86	±	0.81		9.44	±	0.84		9.02	±	0.64
	Ornithine	0.20	±	0.03		**0.48**	**±**	**0.06**		0.33	±	0.04		0.69	±	0.20		1.02	±	0.14		**0.75**	**±**	**0.10**		0.95	±	0.07
	Phenylalanine	0.27	±	0.02		**0.42**	**±**	**0.01**		0.30	±	0.06		0.48	±	0.07		0.38	±	0.02		0.34	±	0.02		0.62	±	0.11
	Proline	14.99	±	1.51		**132.87**	**±**	**25.12**		**22.90**	**±**	**6.70**		31.92	±	10.03		32.83	±	1.98		13.49	±	2.50		72.38	±	21.96
	Serine	11.78	±	0.74		**21.17**	**±**	**1.87**		**10.05**	**±**	**1.50**		14.65	±	1.66		14.97	±	2.30		**19.21**	**±**	**2.43**		21.09	±	1.20
	Threonine	6.19	±	0.57		8.24	±	0.74		**5.41**	**±**	**0.82**		7.47	±	0.82		6.46	±	0.20		**7.96**	**±**	**0.36**		**9.78**	**±**	**0.52**
	Valine	3.16	±	0.46		4.07	±	0.23		**2.55**	**±**	**0.39**		3.85	±	0.16		**3.08**	**±**	**0.14**		2.82	±	0.19		4.12	±	0.55
Sugars and Sugars alcohol	Erythritol	0.36	±	0.04		0.37	±	0.09		0.23	±	0.04		**0.23**	**±**	**0.02**		0.32	±	0.09		**0.17**	**±**	**0.01**		0.28	±	0.06
	Fructose	136.13	±	21.73		210.69	±	32.90		**63.35**	**±**	**9.58**		103.22	±	25.60		37.08	±	5.09		**24.20**	**±**	**2.47**		86.44	±	30.43
	Glucose	248.73	±	26.39		202.63	±	12.96		**136.48**	**±**	**17.44**		**148.94**	**±**	**29.51**		**60.60**	**±**	**6.77**		**45.81**	**±**	**4.40**		103.67	±	31.69
	Myo-inositol	28.29	±	6.13		24.45	±	1.81		24.77	±	2.01		16.50	±	2.95		15.97	±	2.25		**7.51**	**±**	**0.83**		**19.21**	**±**	**1.67**
	Sucrose	106.79	±	16.47		130.84	±	3.34		**97.65**	**±**	**7.05**		92.25	±	6.59		67.91	±	3.99		106.66	±	9.06		133.56	±	41.09
	Trehalose	0.61	±	0.08		0.64	±	0.08		0.52	±	0.04		0.50	±	0.06		0.70	±	0.07		**0.42**	**±**	**0.02**		**0.68**	**±**	**0.12**
	Galactinol	1.67	±	0.32		3.51	±	0.69		**1.16**	**±**	**0.19**		1.00	±	0.43		0.80	±	0.14		0.60	±	0.39		0.87	±	0.13
	Raffinose	1.51	±	0.36		**6.61**	**±**	**1.55**		**1.61**	**±**	**0.43**		1.07	±	0.61		1.23	±	0.18		**0.21**	**±**	**0.05**		**1.73**	**±**	**0.53**
Organic acids	Glycerate	0.80	±	0.05		**1.04**	**±**	**0.03**		**0.78**	**±**	**0.04**		0.82	±	0.06		0.79	±	0.07		**0.93**	**±**	**0.02**		1.63	±	0.31
	Succinate	5.91	±	0.98		5.03	±	1.12		5.05	±	0.37		3.34	±	0.31		3.16	±	0.42		**3.07**	**±**	**0.60**		2.34	±	0.23
	Fumarate	334.15	±	41.23		**185.12**	**±**	**22.57**		**265.33**	**±**	**17.26**		243.02	±	54.80		174.27	±	33.72		**123.66**	**±**	**15.95**		**221.18**	**±**	**33.92**
	Malate	2.81	±	0.11		2.49	±	0.29		2.92	±	0.09		2.65	±	0.33		2.80	±	0.25		**1.80**	**±**	**0.09**		**4.49**	**±**	**0.54**
	GABA	1.28	±	0.12		1.60	±	0.22		1.26	±	0.13		1.59	±	0.31		1.46	±	0.10		1.34	±	0.11		1.56	±	0.25
	Threonate	9.02	±	0.81		10.75	±	0.37		**14.99**	**±**	**1.07**		**12.99**	**±**	**1.39**		14.87	±	1.41		10.64	±	0.52		**17.11**	**±**	**2.02**
	Pyroglutamate	84.07	±	8.36		94.54	±	8.67		116.02	±	23.83		76.42	±	7.03		**124.69**	**±**	**7.50**		81.67	±	6.64		**134.50**	**±**	**17.59**
	Citrate	2.94	±	0.55		1.59	±	0.31		1.25	±	0.07		5.06	±	2.48		1.22	±	0.02		**1.13**	**±**	**0.21**		1.54	±	0.26
Others	Glycerol	9.30	±	1.23		9.32	±	1.42		6.24	±	0.29		10.58	±	2.75		5.50	±	0.42		6.73	±	0.61		8.63	±	2.08
	Putrescine	1.27	±	0.22		1.51	±	0.19		1.14	±	0.23		1.04	±	0.19		1.51	±	0.16		1.49	±	0.13		1.30	±	0.18
	Dehydroascorbate	0.49	±	0.07		0.38	±	0.02		0.36	±	0.03		0.48	±	0.04		**0.36**	**±**	**0.01**		0.34	±	0.01		**0.41**	**±**	**0.01**
	Spermidine	0.16	±	0.03		0.15	±	0.02		0.14	±	0.03		0.15	±	0.01		0.14	±	0.01		**0.24**	**±**	**0.01**		**0.14**	**±**	**0.01**

Supplemental Table S6 – Root elative GC-MS metabolite content in the *A. thaliana* ecotypes under Al treatment (4-week-old). Values are presented by means ± SE (n=4). Bold numbers represent values that were significantly different ($P < 0.05$) when comparing controls with timepoint 0h and treatment values with their respective control. Both determined by Student's *t* test.

	Root	Col																					
	Metabolite	0h	±	error	24h	±	error	24hT	±	error	72h	±	error	72hT	±	error	120h	±	error	120hT	±	error
Amino acids	Alanine	33.10	±	5.83	**66.19**	**±**	**9.37**	56.67	±	7.55	56.01	±	17.32	93.14	±	12.19	25.98	±	4.45	**74.65**	**±**	**12.68**
	β-alanine	0.40	±	0.07	**0.65**	**±**	**0.06**	0.77	±	0.06	0.54	±	0.03	0.58	±	0.05	0.38	±	0.03	0.46	±	0.03
	Aspartate	22.35	±	4.91	14.55	±	1.04	**8.30**	**±**	**0.37**	**6.45**	**±**	**1.00**	6.44	±	0.73	**6.91**	**±**	**0.34**	**3.86**	**±**	**0.55**
	Glutamate	22.36	±	1.17	20.13	±	3.60	23.31	±	5.23	**9.61**	**±**	**1.80**	17.10	±	3.77	**9.59**	**±**	**2.13**	9.64	±	2.57
	Glutamine	0.20	±	0.01	0.25	±	0.03	**0.85**	**±**	**0.20**	**0.55**	**±**	**0.12**	**1.62**	**±**	**0.27**	**1.05**	**±**	**0.25**	1.21	±	0.17
	Glycine	5.30	±	0.50	6.94	±	0.68	6.58	±	1.44	**3.68**	**±**	**0.33**	3.55	±	0.33	**2.48**	**±**	**0.17**	2.50	±	0.24
	Isoleucine	2.06	±	0.13	2.95	±	0.41	4.03	±	0.49	2.74	±	0.34	3.20	±	0.06	2.23	±	0.39	2.62	±	0.03
	Lysine	0.28	±	0.03	**0.57**	**±**	**0.08**	0.65	±	0.12	0.38	±	0.05	0.35	±	0.04	0.24	±	0.02	0.23	±	0.02
	Methionine	0.48	±	0.07	0.45	±	0.06	0.58	±	0.04	**0.25**	**±**	**0.01**	**0.47**	**±**	**0.03**	0.34	±	0.03	**0.44**	**±**	**0.03**
	Proline	21.32	±	6.87	10.41	±	1.96	**22.81**	**±**	**1.80**	6.79	±	0.91	**21.35**	**±**	**1.61**	6.37	±	1.32	**14.64**	**±**	**2.39**
	Serine	18.67	±	1.89	17.98	±	1.74	**29.09**	**±**	**3.79**	**12.65**	**±**	**1.61**	**25.21**	**±**	**1.95**	**12.29**	**±**	**1.93**	15.32	±	1.45
	Threonine	11.26	±	0.62	13.14	±	1.26	**27.48**	**±**	**1.26**	12.04	±	0.44	**21.82**	**±**	**1.22**	12.03	±	1.54	14.81	±	1.01
	Valine	6.36	±	0.35	**10.32**	**±**	**1.31**	**15.84**	**±**	**0.92**	7.88	±	0.51	**12.34**	**±**	**0.26**	7.08	±	1.14	9.27	±	0.25
Sugars and Sugars alcohol	Fructose	180.22	±	35.23	212.43	±	28.75	207.03	±	23.99	103.86	±	14.80	**242.55**	**±**	**10.17**	178.33	±	16.21	**306.59**	**±**	**20.51**
	Fucose	1.85	±	0.15	**2.51**	**±**	**0.16**	2.16	±	0.14	2.30	±	0.15	2.06	±	0.08	1.96	±	0.11	**2.41**	**±**	**0.10**
	Galactose	1.32	±	0.13	2.54	±	0.73	1.74	±	0.22	**0.87**	**±**	**0.14**	1.26	±	0.15	1.32	±	0.68	2.16	±	0.59
	Glucoheptose	3.08	±	0.27	2.67	±	0.05	2.96	±	0.38	3.73	±	0.29	4.20	±	0.33	**6.26**	**±**	**0.32**	6.04	±	0.71
	Glucose	303.68	±	17.12	**536.23**	**±**	**70.85**	354.10	±	17.01	**225.55**	**±**	**19.60**	257.02	±	16.30	**168.98**	**±**	**0.94**	168.01	±	9.20
	Gluc *	0.54	±	0.14	1.37	±	0.72	0.53	±	0.14	0.62	±	0.07	**1.14**	**±**	**0.06**	**0.99**	**±**	**0.06**	**1.77**	**±**	**0.10**
	Gluc.**	0.35	±	0.05	0.35	±	0.07	0.33	±	0.03	0.36	±	0.05	**0.68**	**±**	**0.03**	**0.63**	**±**	**0.04**	**0.86**	**±**	**0.05**
	Myo-inositol	30.39	±	4.32	45.65	±	9.81	67.41	±	9.71	38.44	±	6.18	**95.02**	**±**	**7.85**	34.80	±	5.34	**79.71**	**±**	**9.80**
	Maltose	0.43	±	0.08	0.56	±	0.08	0.88	±	0.13	0.44	±	0.05	**0.70**	**±**	**0.08**	0.28	±	0.01	**0.41**	**±**	**0.01**
	Sucrose	44.93	±	3.99	63.82	±	8.39	56.36	±	1.27	42.65	±	2.34	41.30	±	3.00	**27.93**	**±**	**0.51**	**24.59**	**±**	**1.34**
	Xylose	2.29	±	0.32	**3.78**	**±**	**0.21**	**2.29**	**±**	**0.23**	1.74	±	0.19	2.08	±	0.11	2.22	±	0.11	**3.04**	**±**	**0.06**
Organic acids	2-Oxo-glutarate	0.63	±	0.09	0.40	±	0.06	0.50	±	0.08	**0.14**	**±**	**0.01**	0.17	±	0.02	**0.20**	**±**	**0.01**	0.17	±	0.02
	Adipate	0.57	±	0.07	0.55	±	0.08	0.70	±	0.13	0.43	±	0.03	0.46	±	0.06	**0.31**	**±**	**0.02**	0.34	±	0.04
	Fumarate	4.18	±	0.56	**1.85**	**±**	**0.24**	2.86	±	0.70	**1.63**	**±**	**0.18**	3.35	±	1.00	**1.94**	**±**	**0.55**	3.49	±	0.93
	GABA	11.26	±	2.03	**20.54**	**±**	**2.98**	22.41	±	4.21	8.81	±	1.28	**20.80**	**±**	**1.99**	**5.76**	**±**	**0.56**	**12.57**	**±**	**2.29**
	Glycerate	23.85	±	16.82	8.23	±	2.20	8.73	±	1.70	2.86	±	0.20	13.72	±	5.69	3.27	±	0.76	7.05	±	2.82
	Malate	64.34	±	26.46	15.67	±	0.70	16.31	±	1.91	**5.88**	**±**	**0.88**	16.98	±	5.49	**8.94**	**±**	**1.25**	10.77	±	3.08
	Pyroglutamate	266.33	±	19.09	323.92	±	35.56	**526.62**	**±**	**17.69**	**356.72**	**±**	**16.34**	**516.89**	**±**	**29.28**	**437.71**	**±**	**70.32**	522.99	±	61.65
	Pyruvate	0.62	±	0.10	0.40	±	0.05	0.50	±	0.10	**0.28**	**±**	**0.01**	0.43	±	0.06	**0.25**	**±**	**0.04**	0.42	±	0.09
	Succinate	143.03	±	17.76	99.46	±	2.91	**62.31**	**±**	**3.49**	**71.38**	**±**	**8.25**	76.86	±	9.28	**63.60**	**±**	**2.63**	**84.89**	**±**	**6.21**
Others	Benzyl alcohol	1.47	±	0.09	**3.17**	**±**	**0.53**	2.09	±	0.17	1.58	±	0.06	**1.23**	**±**	**0.06**	**0.81**	**±**	**0.02**	0.80	±	0.08
	Glu.-1,5-lactone	11.91	±	0.19	10.80	±	1.06	11.17	±	0.06	12.69	±	1.05	11.56	±	0.30	12.76	±	0.92	11.36	±	0.27
	Glycerol	27.73	±	2.09	32.33	±	3.74	40.57	±	7.61	**21.49**	**±**	**1.00**	**29.84**	**±**	**1.84**	**17.33**	**±**	**0.96**	**26.04**	**±**	**2.09**
	Glycerol-3-P	0.45	±	0.03	0.50	±	0.07	0.38	±	0.03	**0.53**	**±**	**0.02**	**0.36**	**±**	**0.03**	**0.74**	**±**	**0.07**	**0.30**	**±**	**0.01**
	Guanidine	0.33	±	0.02	2.04	±	1.23	1.11	±	0.20	0.95	±	0.27	0.44	±	0.05	0.42	±	0.05	0.49	±	0.05
	Nicotinic acid	0.58	±	0.06	0.78	±	0.07	1.02	±	0.09	**0.83**	**±**	**0.08**	1.05	±	0.12	**0.96**	**±**	**0.02**	0.77	±	0.12
	Phosphoric acid	34.60	±	4.31	52.49	±	9.07	**27.55**	**±**	**0.96**	43.23	±	7.77	**17.97**	**±**	**2.33**	46.05	±	5.28	**12.00**	**±**	**0.42**
	Putrescine	1.05	±	0.28	**2.72**	**±**	**0.42**	2.70	±	0.69	1.13	±	0.26	1.22	±	0.11	0.47	±	0.07	**1.83**	**±**	**0.05**

Urea	8.41 ± 1.18	**3.21 ± 0.42**	**13.04 ± 3.28**	7.45 ± 0.74	7.74 ± 1.26	5.36 ± 0.81	4.70 ± 0.33

	Root	Ws																					
	Metabolite	0h	±	error	24h	±	error	24hT	±	error	72h	±	error	72hT	±	error	120h	±	error	120hT	±	error
Amino acids	Alanine	23.04	±	4.11	**82.84**	**±**	**8.70**	112.23	±	16.07	61.89	±	15.76	60.41	±	5.85	**47.94**	**±**	**7.44**	45.40	±	2.62
	β-alanine	0.38	±	0.03	0.43	±	0.06	**0.80**	**±**	**0.09**	0.49	±	0.06	0.51	±	0.03	0.33	±	0.02	**0.48**	**±**	**0.04**
	Aspartate	22.76	±	5.63	9.84	±	0.92	8.35	±	0.68	9.99	±	2.19	6.87	±	0.46	**3.31**	**±**	**0.61**	5.21	±	0.53
	Glutamate	14.63	±	2.23	11.40	±	1.78	**27.30**	**±**	**3.80**	12.85	±	3.07	13.78	±	2.37	**5.99**	**±**	**1.09**	10.40	±	1.91
	Glutamine	0.23	±	0.04	0.45	±	0.09	0.64	±	0.09	**0.57**	**±**	**0.09**	1.45	±	0.36	**0.48**	**±**	**0.06**	**1.32**	**±**	**0.28**
	Glycine	5.21	±	0.72	4.10	±	0.37	**5.91**	**±**	**0.56**	3.35	±	0.41	2.93	±	0.16	3.70	±	0.85	2.49	±	0.13
	Isoleucine	2.08	±	0.16	3.01	±	0.45	3.98	±	0.69	2.94	±	0.61	2.45	±	0.19	2.61	±	0.35	2.79	±	0.25
	Lysine	0.31	±	0.03	0.48	±	0.11	0.73	±	0.13	0.23	±	0.04	0.23	±	0.02	**0.17**	**±**	**0.03**	0.24	±	0.02
	Methionine	0.32	±	0.03	0.36	±	0.05	0.48	±	0.04	0.37	±	0.06	0.43	±	0.03	**0.21**	**±**	**0.03**	**0.36**	**±**	**0.01**
	Proline	22.86	±	9.94	13.92	±	3.29	**32.37**	**±**	**6.63**	20.68	±	2.76	19.91	±	1.34	7.79	±	0.94	8.32	±	0.27
	Serine	15.80	±	2.77	18.12	±	3.50	27.15	±	1.78	13.85	±	2.14	**18.89**	**±**	**0.70**	9.73	±	1.72	**14.16**	**±**	**0.35**
	Threonine	13.47	±	1.55	11.24	±	1.38	**21.95**	**±**	**2.52**	14.63	±	2.49	18.30	±	0.68	11.45	±	2.08	15.14	±	0.73
	Valine	7.21	±	0.65	9.65	±	1.93	13.70	±	1.41	9.57	±	1.75	10.08	±	0.42	7.95	±	1.39	9.14	±	0.44
Sugars and Sugars alcohol	Fructose	77.72	±	14.94	**218.65**	**±**	**43.63**	**110.01**	**±**	**13.27**	**301.30**	**±**	**69.50**	192.56	±	14.27	**185.13**	**±**	**26.83**	233.33	±	10.51
	Fucose	1.48	±	0.04	**2.08**	**±**	**0.10**	1.91	±	0.15	**2.38**	**±**	**0.22**	1.84	±	0.11	**2.11**	**±**	**0.21**	2.09	±	0.08
	Galactose	0.75	±	0.15	1.39	±	0.37	1.27	±	0.12	1.40	±	0.32	0.83	±	0.06	0.97	±	0.40	3.24	±	1.38
	Glucoheptose	1.89	±	0.11	**3.40**	**±**	**0.18**	2.53	±	0.40	**4.28**	**±**	**0.27**	**3.60**	**±**	**0.10**	**5.58**	**±**	**0.30**	6.05	±	0.54
	Glucose	264.85	±	58.41	376.48	±	33.57	**289.51**	**±**	**17.03**	331.33	±	34.71	**231.46**	**±**	**20.10**	137.13	±	15.41	155.59	±	3.20
	Gluc *	0.30	±	0.07	**1.35**	**±**	**0.41**	**0.23**	**±**	**0.04**	1.27	±	0.47	0.88	±	0.07	0.74	±	0.19	1.27	±	0.14
	Gluc.**	0.27	±	0.03	0.64	±	0.21	0.22	±	0.02	0.90	±	0.27	0.67	±	0.05	**0.53**	**±**	**0.03**	**0.70**	**±**	**0.03**
	Myo-inositol	20.80	±	3.05	**35.96**	**±**	**1.43**	**98.90**	**±**	**1.70**	**45.89**	**±**	**8.94**	**103.84**	**±**	**10.22**	**61.33**	**±**	**3.06**	66.83	±	1.82
	Maltose	0.39	±	0.07	0.49	±	0.09	**0.84**	**±**	**0.04**	**0.70**	**±**	**0.09**	0.75	±	0.01	0.30	±	0.03	**0.55**	**±**	**0.01**
	Sucrose	39.01	±	8.97	60.88	±	1.03	**55.56**	**±**	**1.73**	49.30	±	4.40	38.35	±	4.11	19.91	±	2.19	23.01	±	0.49
	Xylose	1.48	±	0.09	**2.32**	**±**	**0.26**	1.64	±	0.15	**2.92**	**±**	**0.47**	1.91	±	0.15	**2.07**	**±**	**0.09**	2.47	±	0.07
Organic acids	2-Oxo-glutarate	0.34	±	0.00	**0.53**	**±**	**0.00**	**0.40**	**±**	**0.02**	**0.24**	**±**	**0.04**	0.18	±	0.03	**0.19**	**±**	**0.02**	0.20	±	0.01
	Adipate	0.54	±	0.03	**0.41**	**±**	**0.04**	0.53	±	0.06	0.43	±	0.05	0.45	±	0.03	**0.23**	**±**	**0.02**	**0.30**	**±**	**0.01**
	Fumarate	2.70	±	0.27	2.20	±	0.38	1.64	±	0.24	5.75	±	2.20	2.07	±	0.11	**1.27**	**±**	**0.10**	1.72	±	0.17
	GABA	8.82	±	1.42	14.39	±	2.71	**32.57**	**±**	**3.44**	9.84	±	1.96	**16.77**	**±**	**0.40**	10.48	±	1.83	12.79	±	1.84
	Glycerate	9.99	±	2.11	5.59	±	1.16	6.18	±	1.51	**4.16**	**±**	**0.56**	**11.57**	**±**	**2.02**	3.55	±	1.51	8.14	±	2.25
	Malate	48.38	±	14.86	16.45	±	2.23	11.32	±	1.13	13.35	±	2.05	17.95	±	2.00	**9.64**	**±**	**2.13**	12.74	±	1.65
	Pyroglutamate	223.23	±	30.11	336.66	±	48.50	**476.94**	**±**	**18.87**	**325.53**	**±**	**19.27**	**451.99**	**±**	**23.38**	286.93	±	32.40	**540.18**	**±**	**29.66**
	Pyruvate	0.34	±	0.08	0.36	±	0.04	0.42	±	0.05	0.29	±	0.03	**0.45**	**±**	**0.04**	0.30	±	0.02	**0.41**	**±**	**0.03**
	Succinate	108.48	±	7.32	101.44	±	19.03	58.22	±	2.06	87.63	±	6.09	75.12	±	4.44	**78.42**	**±**	**6.23**	72.73	±	2.62
Others	Benzyl alcohol	1.46	±	0.10	2.03	±	0.28	1.67	±	0.06	1.78	±	0.18	**1.15**	**±**	**0.12**	**0.63**	**±**	**0.08**	0.73	±	0.02
	Glu.-1,5-lactone	7.33	±	0.33	**12.24**	**±**	**0.95**	10.05	±	1.45	**14.27**	**±**	**1.01**	12.19	±	0.18	**12.64**	**±**	**1.07**	13.66	±	0.39
	Glycerol	25.06	±	2.44	23.00	±	0.65	**31.95**	**±**	**2.23**	24.66	±	1.75	25.38	±	0.56	21.61	±	0.82	**26.60**	**±**	**0.85**
	Glycerol-3-P	0.45	±	0.05	0.51	±	0.03	**0.38**	**±**	**0.02**	0.46	±	0.04	**0.36**	**±**	**0.01**	0.54	±	0.05	**0.34**	**±**	**0.02**
	Guanidine	0.26	±	0.04	0.38	±	0.09	0.39	±	0.09	**0.56**	**±**	**0.08**	0.41	±	0.03	0.33	±	0.06	0.32	±	0.04
	Nicotinic acid	0.66	±	0.12	0.63	±	0.02	**0.91**	**±**	**0.06**	0.94	±	0.08	1.04	±	0.02	0.79	±	0.14	0.93	±	0.02
	Phosphoric acid	32.42	±	8.60	46.96	±	7.29	**27.87**	**±**	**2.96**	27.71	±	3.70	**16.57**	**±**	**0.87**	29.26	±	5.89	**15.34**	**±**	**1.36**
	Putrescine	1.10	±	0.36	1.03	±	0.23	1.48	±	0.08	0.96	±	0.12	1.06	±	0.20	0.57	±	0.08	**1.44**	**±**	**0.15**
	Urea	6.02	±	1.43	4.89	±	0.83	**9.03**	**±**	**0.93**	5.15	±	0.54	**8.61**	**±**	**1.08**	6.79	±	1.43	5.67	±	0.55

	Root	Ler																				
	Metabolite	0h	±	error	24h	±	error	24hT	±	error	72h	±	error	72hT	±	error	120h	±	error	120hT	±	error
Amino acids	Alanine	29.82	±	1.84	**53.41**	**±**	**11.21**	49.16	±	5.05	57.75	±	7.57	53.88	±	3.39	31.54	±	4.41	45.13	±	5.58
	β-alanine	0.41	±	0.06	0.39	±	0.03	**0.55**	**±**	**0.04**	0.36	±	0.02	0.40	±	0.03	0.38	±	0.06	0.39	±	0.06
	Aspartate	7.28	±	1.77	4.35	±	1.16	6.42	±	0.59	**2.38**	**±**	**0.21**	2.19	±	0.16	6.07	±	2.09	1.51	±	0.26
	Glutamate	12.05	±	3.63	9.70	±	2.19	15.03	±	2.41	11.17	±	3.62	7.55	±	1.90	5.53	±	2.35	5.57	±	1.08
	Glutamine	0.26	±	0.02	**0.66**	**±**	**0.08**	0.73	±	0.11	**0.84**	**±**	**0.06**	0.62	±	0.08	0.50	±	0.18	0.50	±	0.13
	Glycine	3.80	±	0.61	3.57	±	0.43	4.50	±	0.53	4.00	±	0.38	**2.70**	**±**	**0.15**	3.03	±	0.16	2.41	±	0.35
	Isoleucine	1.93	±	0.19	**3.24**	**±**	**0.42**	2.60	±	0.12	**2.96**	**±**	**0.23**	2.66	±	0.24	2.28	±	0.18	2.31	±	0.19
	Lysine	0.28	±	0.06	0.50	±	0.10	0.35	±	0.06	0.33	±	0.04	0.23	±	0.03	0.24	±	0.03	0.17	±	0.00
	Methionine	0.38	±	0.03	**0.63**	**±**	**0.10**	0.40	±	0.03	0.35	±	0.02	0.27	±	0.05	7.31	±	4.47	0.30	±	0.01
	Proline	6.84	±	0.89	**28.81**	**±**	**2.38**	**11.95**	**±**	**0.26**	12.99	±	2.75	13.58	±	3.77	4.49	±	0.36	**10.23**	**±**	**0.98**
	Serine	12.24	±	2.34	16.54	±	3.28	16.35	±	1.14	11.32	±	0.55	10.77	±	1.50	7.54	±	1.57	6.71	±	0.39
	Threonine	11.50	±	1.49	17.08	±	2.83	24.32	±	0.30	13.10	±	0.60	**18.56**	**±**	**1.48**	21.48	±	5.01	11.49	±	0.63
	Valine	7.14	±	0.88	**11.79**	**±**	**1.45**	9.69	±	0.34	9.43	±	0.59	10.88	±	1.14	5.96	±	0.32	**8.62**	**±**	**0.88**
Sugars and Sugars alcohol	Fructose	136.16	±	18.54	**360.68**	**±**	**23.85**	**227.00**	**±**	**17.84**	**350.42**	**±**	**12.19**	**195.60**	**±**	**17.74**	**294.45**	**±**	**48.64**	237.57	±	11.16
	Fucose	1.44	±	0.13	1.60	±	0.04	1.67	±	0.05	**1.85**	**±**	**0.04**	**1.53**	**±**	**0.03**	**2.07**	**±**	**0.20**	**1.61**	**±**	**0.06**
	Galactose	1.22	±	0.35	1.79	±	0.14	**1.02**	**±**	**0.04**	**4.48**	**±**	**0.80**	2.22	±	0.90	2.84	±	1.25	0.95	±	0.09
	Glucoheptose	1.98	±	0.27	3.10	±	0.44	2.97	±	0.14	**3.77**	**±**	**0.61**	2.56	±	0.60	2.78	±	1.09	3.97	±	0.48
	Glucose	359.44	±	34.96	277.37	±	12.54	302.97	±	20.56	**216.78**	**±**	**13.86**	**258.38**	**±**	**6.40**	**173.85**	**±**	**6.36**	164.16	±	8.29
	Gluc *	0.59	±	0.19	1.05	±	0.20	1.59	±	0.22	0.96	±	0.16	**0.37**	**±**	**0.06**	9.17	±	4.84	1.29	±	0.15
	Gluc.**	0.27	±	0.04	**0.90**	**±**	**0.14**	**0.51**	**±**	**0.06**	**0.82**	**±**	**0.03**	**0.40**	**±**	**0.03**	1.97	±	1.25	0.66	±	0.03
	Myo-inositol	30.27	±	2.69	**62.19**	**±**	**13.39**	85.25	±	8.94	**61.54**	**±**	**7.42**	**39.94**	**±**	**4.08**	66.80	±	24.19	31.07	±	2.53
	Maltose	1.41	±	0.71	0.45	±	0.08	0.50	±	0.07	0.49	±	0.04	0.39	±	0.03	0.73	±	0.22	0.38	±	0.03
	Sucrose	47.23	±	2.69	41.87	±	1.47	**59.63**	**±**	**2.51**	**32.42**	**±**	**2.51**	**45.87**	**±**	**2.38**	**13.65**	**±**	**5.68**	24.39	±	1.59
	Xylose	1.66	±	0.23	2.22	±	0.13	**1.60**	**±**	**0.05**	2.12	±	0.08	**1.54**	**±**	**0.09**	3.56	±	1.10	2.01	±	0.14
Organic acids	2-Oxo-glutarate	0.32	±	0.01	0.20	±	0.05	0.23	±	0.03	0.22	±	0.10	0.36	±	0.01	**0.20**	**±**	**0.04**	0.09	±	0.02
	Adipate	0.27	±	0.02	0.30	±	0.04	0.40	±	0.04	0.24	±	0.01	0.29	±	0.02	0.33	±	0.08	0.24	±	0.02
	Fumarate	4.54	±	0.88	2.80	±	0.57	3.45	±	0.12	3.31	±	1.21	4.59	±	1.19	**1.56**	**±**	**0.32**	2.88	±	0.71
	GABA	8.74	±	1.89	**15.80**	**±**	**1.49**	15.20	±	1.13	**15.47**	**±**	**1.68**	14.56	±	2.04	7.93	±	0.05	**11.64**	**±**	**1.33**
	Glycerate	5.28	±	1.84	3.41	±	1.82	3.01	±	0.03	1.38	±	0.12	1.88	±	0.31	5.29	±	2.31	1.54	±	0.24
	Malate	17.25	±	3.49	9.03	±	2.46	**15.18**	**±**	**0.94**	**4.51**	**±**	**0.44**	4.58	±	0.46	**5.35**	**±**	**1.26**	5.81	±	0.94
	Pyroglutamate	213.40	±	5.61	254.20	±	38.52	347.45	±	7.76	263.48	±	20.13	218.63	±	11.36	206.82	±	90.59	194.60	±	22.19
	Pyruvate	0.39	±	0.05	0.48	±	0.18	**0.98**	**±**	**0.06**	0.48	±	0.04	0.67	±	0.09	0.49	±	0.07	0.53	±	0.09
	Succinate	66.84	±	7.27	70.45	±	11.81	77.74	±	3.65	67.24	±	1.87	56.74	±	5.41	57.81	±	15.80	62.68	±	6.31
Others	Benzyl alcohol	1.85	±	0.09	**1.36**	**±**	**0.16**	1.81	±	0.12	**0.99**	**±**	**0.07**	**1.33**	**±**	**0.08**	**0.75**	**±**	**0.06**	0.90	±	0.08
	Glu.-1,5-lactone	7.47	±	0.21	9.46	±	1.43	10.46	±	0.57	**8.93**	**±**	**0.44**	8.68	±	0.57	**11.25**	**±**	**0.46**	**8.29**	**±**	**0.29**
	Glycerol	22.47	±	3.40	23.47	±	1.95	**30.50**	**±**	**0.03**	25.15	±	1.79	25.95	±	0.84	100.19	±	48.29	23.88	±	2.09
	Glycerol-3-P	0.30	±	0.05	0.34	±	0.06	0.38	±	0.02	0.28	±	0.04	0.28	±	0.06	0.48	±	0.11	**0.20**	**±**	**0.02**
	Guanidine	0.38	±	0.13	0.33	±	0.02	0.62	±	0.22	0.36	±	0.05	0.26	±	0.05	0.43	±	0.07	0.90	±	0.18
	Nicotinic acid	0.24	±	0.03	**0.31**	**±**	**0.01**	**0.57**	**±**	**0.03**	0.25	±	0.01	**0.39**	**±**	**0.02**	2.23	±	1.22	0.34	±	0.01
	Phosphoric acid	22.38	±	5.75	13.95	±	2.25	13.81	±	0.34	14.95	±	1.28	**9.86**	**±**	**0.31**	32.56	±	7.29	**8.37**	**±**	**0.67**
	Putrescine	2.00	±	0.71	3.28	±	0.20	**0.94**	**±**	**0.08**	3.17	±	0.13	**1.61**	**±**	**0.19**	0.39	±	0.06	**1.48**	**±**	**0.31**
	Urea	4.26	±	1.05	3.56	±	0.23	**8.67**	**±**	**1.53**	5.33	±	0.76	8.91	±	1.77	4.92	±	1.19	5.24	±	0.47

*(2-amino-2-deoxy); **(2-deoxy)