Graphical Abstract

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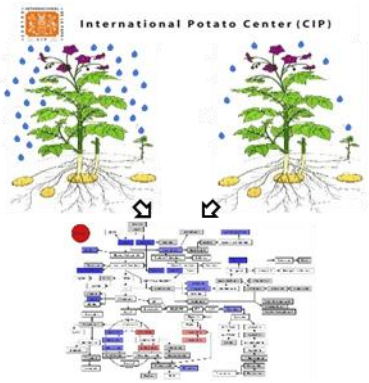

Physiological and metabolic adaptations were integrated to elucidate the difference in tolerance/ susceptibility to water restriction between five potato clones.

Highlights: (85 characters each)

- Majority of metabolic adaptations were observed in the leaf tissue.
- Late onset of changes in chlorophyll and phenolics confers higher tolerance.
- Genetic composition can overcome the environmental effect.
- Metabolite profiling highlighted distinct metabolic signatures per genotype.

Identification of metabolites associated with water stress responses in *Solanum tuberosum* L. clones

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Abstract (200 words)

Water deficiency has become a major issue for modern agriculture as its effects on crop yields and tuber quality have become more pronounced. Potato genotypes more tolerant to water shortages have been identified through assessment of yield and dry matter. In the present study, a combination of metabolite profiling and physiological/agronomical measurements has been used to explore complex system level responses to non-lethal water restriction. The metabolites identified were associated with physiological responses in three different plant tissues (leaf, root and tuber) of five different potato genotypes varying in susceptibility/tolerance to drought. This approach explored the potential of metabolite profiling as a tool to unravel sectors of metabolism that react to stress conditions and could mirror the changes in the plant physiology. The metabolite results showed different responses of the three plant tissues to the water deficit resulting either in different levels of the metabolites detected or different metabolites expressed. The leaf material displayed the most changes to drought as reported in literature. The results highlighted genotype–specific signatures to water restriction over all three plant tissues suggesting that the genetics can predominate over the environmental conditions. This will have important implications for future breeding approaches.

Keywords: *Solanum tuberosum* L., Solanaceae, metabolite profiling, plant response, water restriction, tolerance, phenolic compounds

1. Introduction

Our changing climate, with more frequent fluctuations in temp. and precipitation levels, are affecting the yield and quality of crops, such as potato, one of the most important crops in the world (Bates et al., 2008). The limitation of water, like many other abiotic stresses, is a particular issue in many crop plants and mobilises multiple sectors of the plant metabolism as part of its stress response. Hence, presently it is difficult to associate a single key metabolite with the environmental adaptation to abiotic stresses (André et al., 2009; Sánchez-Rodríguez et al., 2011). The unpredictability of weather conditions, especially at critical periods such as plant emergence and tuberisation, can affect the plant development and lead to dramatic yield losses (Martínez et al., 1992; Obidiegwu et al., 2015). This emphasises the need for improved potato varieties that can withstand those environmental stresses (Evers et al., 2010).

Tolerance to water limitation in plants is a well-studied topic and has been intricately linked to photosynthesis and carbohydrate metabolism (Zingaretti et al., 2013). The response pattern of plants to water restriction is regulated by the intensity, duration and rate of progression of imposed water restriction and consists of a combination of physiological and cellular adaptations (Jefferies et al., 1993; Obidiegwu et al., 2015).

Most of the water restriction related changes can be detected in the leaves, the energy production organ of plants. One common response in leaves under mild to moderate water limitation is to lower their stomatal conductance, thus helping to maintain the water balance of the plant. Under prolonged water restriction, the cell turgor is affected despite the stomatal closure which can be counteracted through osmotic adjustment. Thus, the osmotic potential of the cell is lowered through accumulation of osmolytes

such as amino acids, sugars and polyols inside the cell (Evers et al., 2010; Morgan, 1984; Zingaretti et al., 2013). A side effect of stomatal closure is a reduction in net photosynthesis due to limited intracellular CO_2 levels (Chaves et al., 2003) which in consequence leads to an increase in photorespiration, a lower carbon fixation and ultimately in a reduced partitioning of assimilates affecting the tuber initiation and bulking (Loon, 1981).

Water limitation, equal to many stress conditions, causes a rise of reactive oxygen species (ROS) which can lead to oxidative damage within the cells. Genes implicated in the detoxification of ROS were identified as up-regulated in potato upon drought stress (Watkinson et al., 2008). Most of these genes are responsible for increased production of metabolites (e.g. phenylpropanoids), with antioxidant properties, able to scavenge ROS (Cruz de Carvalho, 2008; Dixon et al., 1995).

Metabolite profiling approaches have the potential to provide valuable phenotypic information on a broad range of metabolites across metabolic pathways that contribute to plant processes and responses to external stimuli or stresses (Jespersen et al., 2015). Capturing metabolite levels over a resolved time-course, can provide successive snapshots of the metabolism which provides a dynamic overview of the current metabolic status of the plant. This methodology is useful in investigating the complex interacting mechanisms of cellular metabolic pathways in response to perturbations such as drought stress (Kim et al., 2007).

The need for more tolerant potato varieties that can withstand fluctuation in water availability and still deliver good yields and quality has becomes the focus of modern breeding programs. For a better understanding of the different drought sensitivity of five

potato breeding clones, those clones were subjected to water restriction followed by a metabolite profiling approach to establish global changes arising across sectors of metabolism. The present study focused on untargeted and targeted analysis of metabolite features in leaves at 30 days after water restriction initiation (DAWRI) and leaves, roots and tubers at 70 DAWRI of five potato genotypes under a control treatment (CT) and water restriction (WR). Additionally, physiological properties of the plants, including osmotic potential (OP), photosynthetic activity (A_{sat}) and several others, were measured to monitor adaptive responses of the clones. Collectively, these data will enable (I) the assessment of the different responses to water restriction throughout the plant, (II) link physiological and metabolic properties and (III) connect the degree of tolerance to key metabolites and/or biochemical pathways.

2. Results and Discussion

2.1 Physiological and agronomical measurements defining tolerance/susceptibility

The WR treatment was started 28 days after sprout transplanting in order to expose the plants to water stress during tuberisation and bulking and evaluate the resulting metabolic changes throughout the potato plant. PCA analysis was used to identify the physiological traits with the greatest contribution to the five genotypes varying in tolerance to drought. Physiological traits with the highest eigenvalues (top 15%) were considered to have the greatest impact for a component. The biplot (Supplementary Fig.1B) of physiological/agronomical data (loadings) and genotypes (scores) highlighted a clear clustering between CT and WR treatments. The loadings HI of dry and fresh weight, CCI at all time points and OP at 0 to 15 DAWRI were associated with the WR cluster and the loadings biomass of fresh and dry weight, A_{sat} and g_s at 15 DAWRI and RWC at 35 and 70 DAWRI were correlated with the CT cluster. This was to be expected as well-watered plants have more excess water available (RWC) leading to “normal” plant performance /biomass production, whereas plants under water-stress focus their intracellular processes on tuber development instead of leaf production resulting in a higher HI (Obidiegwu et al., 2015).

For agronomical purposes, it is important that the plant maintains tuber formation and development despite the water dependent reduction in photosynthetic capacity (Legay et al., 2011). Therefore, biomass and HI define the DTI and are distinct indicators to assess differences in tolerance between potato clones (Table 3) (Cabello et al., 2013). The clones, investigated in this study, were chosen for their abiotic stress properties and showed similar HI to previous studies (e.g. Ramírez et al., 2015a). All clones had

higher HI values under WR and according to those same values, CIP397077.16 had the best performance followed by CIP390637.1, CIP392797.22, CIP394611.112 and CIP395448.1. Interestingly, CIP397077.16 showed no significant difference between the HI of dry and fresh weight under WR and CT conditions. The total biomass at 70DAWRI showed that the clones with lower HI values, CIP392797.22, CIP394611.112 and CIP395448.1, weighed more than the two better performing clones. Hence, the DTI was calculated to establish a precise ranking of tolerance of the five clones investigated. The DTI values (Supplementary Fig.1A) described CIP397077.16 as the most tolerant followed by CIP392797.22, CIP394611.112, CIP390637.1 and CIP395448.1. In this respect, the DTI results for CIP395448.1 differed from the expected high tolerance properties established in the breeding program (Table 2) (Mihovilovich et al., 2015). This indicated that under the growth conditions of the present study the CIP395448.1 clone could not use the available water as effectively as reported previously (Ramírez et al., 2015b).

The underlying metabolic processes of the leaf, tuber and root material were evaluated with untargeted analysis, revealing between 3000 and 7000 metabolite features, and with targeted analysis, identifying ~60 compounds including primary (e.g. glucose, malic acid) and secondary metabolites (e.g. carotenoids, phenolic compounds). The untargeted analysis was visualised as a PCA plot (Fig. 1) and highlighted the environmental conditions (CT and WR) as the main differentiator within each of the three plant parts, as seen for the physiological/agronomical data (Supplementary Fig.1). This corroborates that the metabolome is linked to the physiological properties and vice versa (Peng et al., 2015). The least separation was detected within the root material

(Fig. 1D). CIP395448.1, the only clone with a BW population background, showed a separate cluster from the other four clones within both treatments. This indicated significant differences in the metabolic composition. For a more detailed view of the clustering patterns, PCA analysis was performed of solely CT or WR treated leaf samples at 30 and 70 DAWRI (Supplementary Fig.2). The clusters of the clones within each treatment had the same trends as the combined score plots (Fig. 1A, B). However, leaves under WR at 70 DAWRI showed the least pronounced cluster (Supplementary Fig.2D), which suggested similar metabolic adaptations under stress conditions (e.g. accumulation of phenylpropanoids (Wegener et al., 2015)) despite the genotypic differences. The PCA analysis of leaves and tuber showed a general trend of clusters (Fig. 1A, B) and indicated a similar metabolic composition of the tolerant clone CIP397077.16 and CIP390637.1. Even though, the latter clone showed a lower DTI than expected. This would suggest that the majority of metabolic changes activated under WR are the same between the two clones and a few specialised pathways influenced the better performance of the tolerant clone CIP397077.16. The cluster of the medium-tolerant clone CIP392797.22 was located closely to the cluster of the latter two clones. The sensitive clone CIP394611.112 of the LTVR population clustered the furthest away from the other three LTVR clones and was closest to CIP395448.1, the other sensitive clone with BW population background. This is an interesting finding insofar, as the metabolic differences of the two sensitive clones could be influenced by the different backgrounds and their metabolic similarities could be related to metabolic traits causing the susceptibility to the WR condition.

The metabolic changes, specific to each clone under WR conditions, were elucidated through the more detailed targeted analysis. For this purpose, a direct comparison of metabolite levels under WR and CT was performed and revealed a variety of changes and similarities between genotypes and population background (Fig. 2) which will be discussed hereafter in connection with physiological traits.

2.2 Photosynthetic activity and chlorophyll content under WR

The chlorophyll content was measured as CCI in SPAD units and together with other photosynthesis related isoprenoids by UPLC. The SPAD units showed significant differences between clones at all measurements (0, 15, 25 and 70 DAWRI), but statistical differences between treatments were evidenced at 25 DAWRI, and genotype by treatment interaction at 70 DAWRI (Supplementary File 1). In average, CCI of plants subjected to WR were significantly greater than those of the CT at all times points (Table 3), even before the WR treatment was initiated (0 DAWRI), which need to be taken into account for the evaluation of further time points. The CCI values for both plant treatments increased until the last measurement at 70 DAWRI with the exception of the two tolerant clones (CIP397077.16 and CIP390637.1). Both these clones had significantly decreased CCI values under WR conditions, whilst the other clones retained higher values than their controls. All breeding clones but the tolerant clone CIP397077.16 increased their CCI values significantly under WR at 25 DAWRI. The two susceptible clones CIP394611.112 and CIP395448.1 also showed a significant increase in CCI values at 25 DAWRI and maintained the increased CCI values until 70 DAWRI.

The UPLC measurements at 35 DAWRI showed no significant change of any of the photosynthesis related isoprenoids for the four LTVR clones and for CIP395448.1

decreased levels were detected of antheraxanthin and two violaxanthin isomers by less than 0.1 fold. At 70 DAWRI, CIP395448.1 showed no change of any isoprenoids, whereas the four LTVR clones showed significantly decreased levels of chlorophyll *a* and *b*. Only the genotypes of the LTVR population showed increased levels of phytoene, a carotenoid precursor, by ~1.1 fold at 35 and 70 DAWRI. This could indicate a switch of GGPP utilisation from the predominant use for chlorophyll synthesis to carotenoid synthesis (Zingaretti et al., 2013) and correlates to downregulated genes for chlorophyll synthesis (Legay et al., 2011).

A comparison of the two chlorophyll measurements indicated very different results. However, certain trends of those results become visible when both analysis are used for the interpretation of adaptations under WR. The adjustment of the photosynthesis processes is directly linked to the utilisation of available water resources and the carbon allocation to the tuber (Obidiegwu et al., 2015). The chlorophyll content seemed to be the determining factor for the photosynthesis processes in the clones studied, as both A_{sat} and g_s, known to be lowered under drought to conserve water (Obidiegwu et al., 2015), did not change for any clone at any time point measured. The mid-tolerant clone CIP392797.22 had a similar chlorophyll/violaxanthin profile to the tolerant CIP397077.16 and also showed a decrease of CCI from 25 to 70DAWRI. This might be a factor for the better performance of CIP392797.22 than the reportedly tolerant CIP390637.1, which showed a decrease of CCI but far less change of chlorophyll levels. Furthermore, the chlorophyll content could be a deciding factor for the metabolic difference of the two susceptible clone. Even though both showed the same trends of CCI values, the lack of change in chlorophyll levels of CIP395448.1 probably led to a

quicker consumption of already limited water, enhancing the drought stress on the plant and reducing the tuber formation or development (Zingaretti et al., 2013).

2.3 OP and RWC and related metabolites affecting tuber formation

Significant differences in RWC between treatments were detected at 35 DAWRI with an average RWC decrease of 5% under WR and a 7% increase under CT (Table 3). This trend continued up to 70 DAWRI and showed statistical differences between breeding clones (Supplementary File 1). WR samples showed a further 18% decrease at 70 DAWRI resulting in RWC values between 60-70%, whereas CT samples maintained RWC values above 90%. Interestingly, the OP only showed difference between treatments at 15 DAWRI and remained similar for the remaining experiment between control and stressed plants and between genotypes (Table 3). CIP394611.112 under WR showed statistically lower OP values than under CT at 15 DAWRI and maintained decreasing OP values until the last measurement. The other four clones maintained their cellular pressure under WR treatment (Supplementary File 1).

On the biochemical level, most of the metabolite changes associated with drought responses (e.g. sugar and osmolytes accumulation (Zingaretti et al., 2013)) were detected in the leaves of the LTVR accessions. The metabolite changes common to all five genotypes in leaf were a decrease of citric acid and increases of gluconic acid, ribitol and metabolites of the phenylpropanoid pathway, in particular phenylalanine and naringin. The increase of naringin was above 2.5 fold for all genotypes at 70 DAWRI. The highest increase of naringin in response to water restriction was detected in CIP397077.16 by 68.2 fold at 70 DAWRI, followed by CIP390637.1 and CIP394611.112 with a 37.8 and 28.1 fold increase, respectively. The increase of phenylalanine and the

decrease of citric acid was less at 70 DAWRI than at 30 DAWRI for all genotypes except CIP397077.16. The lack of change in osmolytes levels and reduced levels of phenolic compounds in the sensitive clone CIP395448.1 appears to be a contributing factor to this clone's comparative inability to adapt to drought conditions. The lack of osmolytes and phenolic compounds under water deficiency leads to a deformation of the transportation system of the plant (xylem) and further interferes with its ability to retain water from the soil (Zingaretti et al., 2013). These phenotypic effects were observed for the sensitive clone CIP395448.1, as both the OP and RWC values, displaying cellular pressure and turgidity, were reduced at 25 and 70DAWRI. The second highest reduction of RWC and OP values was observed for the other sensitive clone CIP394611.112. Contrary to this, one of the most distinct features of the tolerant clone CIP397077.16 was a delayed increase of phenylpropanoid formation in the leaves. This delayed increase of phenolic compounds in combination with the induction of one specific phenolic compound (naringin, a glycoside of naringenin) could infer a more efficient ROS scavenging mechanism of this genotype under drought. Activation of naringenin chalcone synthase has been shown to play a particular role for tolerance of potato plants to stress conditions (Vasquez-Robinet et al., 2008). Furthermore, the high levels of phenolic ROS scavengers could delay leaf senescence induced by oxidative stress and aid the photosynthetic processes leading to a better carbon fixation and resource allocation to the tuber which corresponds to the better DTI performance of this clone compared to the others (Obidiegwu et al., 2015; Rivero et al., 2007; Schippers et al., 2007).

Another metabolic trait related to tuber formation/development is the allocation and conversion of sucrose to starch. Within the leaf samples, the clones of the LTVR population showed increased levels of saccharides such as fructose, glucose, inositol and ribitol of which most had an above 2.5 fold increase at 70 DAWRI. No change of sucrose levels was detected in leaf of any of the genotypes. The components of the TCA cycle from succinic to malic acid were increased from ~2.0 to above 2.5 fold at 30 and/or 70 DAWRI, contrary to the 0.5 fold decrease of citric and isocitric acid. The clone CIP395448.1 showed very little metabolite changes, which included a decrease of citric and isocitric acid by 0.6-0.7 fold at 30 DAWRI and a 1.7 fold increase of ribitol and 0.7 fold decrease of mannose at 70DAWRI. The metabolite levels in the LTVR clones suggested an active turn-over from fructose to the TCA cycle which was reported for a resistant accession as a result of higher mitochondrial metabolic activity (Vasquez-Robinet et al., 2008). Within the tuber samples, no change of glucose/fructose or intermediates of the TCA cycle could be seen (Fig. 3). These leaf and tuber results suggest that only the yield but not the development of the tuber was affected, as seen by the biomass/HI results and previously described for an acclimated accession of *Solanum tuberosum ssp. andigena* (Watkinson et al., 2008).

2.4 Phenylpropanoids and other metabolites in tuber

Stress-induced metabolism can vary between different plant parts (Dixon et al., 1995) and the range of metabolites identified in tubers, 45 in total (Fig. 3), varied as expected from the leaf material. The intermediates of the phenylpropanoid pathway varied not only in content but also in composition between the leaves, tuber and roots. The three phenylpropanoids, naringenin, rutin and umbelliferone, detected with increased levels in

the leaf material of WR treated plants, showed no change or even decreases in the root material. An exception was the susceptible clone CIP395448.1 which showed an increase of naringin in the roots. This metabolic adaptation could be related to the clone's BW resistance as naringin and other flavonoids in roots are known to confer antimicrobial properties against soil-borne bacteria (Szoboszlay et al., 2016). In tuber, only umbelliferone was detected next to other phenolic compounds such as chlorogenic acid, as reported previously (Navarre et al., 2013). The genotypes CIP397077.16 and CIP395448.1 had increased levels of caffeic acid, ferulic acid, chlorogenic and cryptochlorogenic acid. CIP392797.22 only had increased levels of caffeic and ferulic acid, whereas the remaining two clones showed no change in phenylpropanoids. The importance of phenolic compounds, despite the different expression in the three plant parts, was shown through the phenylalanine content which was induced (2.6 to 4 fold) in all genotypes in leaf and tuber and supports the hypothesis that drought stress is perceived differently in each plant part and activates tissue specific regulators (Dixon et al., 1995; Sánchez-Rodríguez et al., 2011).

Amino acids were the second metabolite group with significant changes in tuber. Glutamine, a central component in plant nitrogen metabolism, was increased by 2.3 to 3.0 fold in four of the five genotypes, whereas CIP394611.112 produced proline at 25.5 fold under WR conditions. Those ratios of both glutamine and proline were not detected in the leaf material. Other amino acids, including leucine, valine, threonine, isoleucine, methionine, tryptophan and serine, were increased in CIP392797.22, CIP394611.112 and CIP390637.1 by 1.9 to 7.4 fold changes and in the case of CIP390637.1 leucine showed an increase by 15.3 fold. Only the changes of tryptophan were consistent

between tuber and leaf material at 70 DAWRI. These changes of amino acids in combination with phenylpropanoid results indicated a negative correlation between the content of free amino acids and phenolic compounds in tuber (Fig. 3). This could be related to induced protein degradation due to lack of ROS protection (Krasensky et al., 2012) or an increase of free amino acids acting as osmolytes in addition to the ROS protection through phenylpropanoids (Yancey, 2001).

In general and contrary to the leaf material, hardly any changes of saccharides were detected in tubers under WR compared to CT conditions. Inositol, relevant for osmoprotection, was increased in the LTVR clones by a fold change of ~2.4 for CIP392797.22 and CIP394611.112 and ~5.9 fold in CIP390637.1 and CIP397077.16. The clone CIP395448.1 showed no change of inositol levels, but had a 1.6 fold increase of another saccharide, sorbose. Overall, CIP395448.1 tuber material showed the least metabolic changes under WR and differed very little in the metabolite levels from the tolerant clone CIP397077.16, even though their leaf material showed significant differences.

2.5 Tolerance properties affecting tuber quality

One aspect that has to be taken into consideration by breeders producing more tolerant potato varieties, is the change in sensory attributes of the tuber such as taste and texture. In this study, the distinct metabolic features of clone CIP397077.16 highlighted genetic traits which can overcome the water stress environment more effectively compared to the other genotypes. These metabolic features included the late onset of changes in chlorophyll and phenylpropanoid levels in leaf and increased levels of phenylpropanoid in tuber. The disadvantage of these increased levels in tuber is that

most phenylpropanoids are known to confer bitterness taste properties (Ripoll et al., 2014). This has been the unforeseen consequences of various breeding programs where the focus of biotic and abiotic resistance has precluded consideration of consumable traits preventing the uptake of the varieties into real life scenarios. The clone CIP392797.22 would be a suitable compromise between harvest loss and taste properties. This clone showed the second best DTI, a higher total biomass and no changes of phenylpropanoid levels in the tuber.

As illustrated in the present study, the use of metabolite profiling provides detection of potential taste/aroma influencing compounds within accessions. The incorporation of these technique into characterisation of germplasm will enable breeding programs to pyramid multiple traits for resistance, productivity and also consumer preferences.

3. Concluding remarks

The present study showed that the successful adaptation to water deficit displayed by several of the selected clones does have a measurable biochemical component. The adaptation processes are complex and require the interaction of several key pathways to maintain key cellular processes necessary for “normal” plant development/growth. The metabolite profiling highlighted that a distinct metabolic signature can only really be seen in a comparison to other clones as adaptation to drought stresses involves multi-pathway adaptation rather than the introduction or presence of one novel compound/marker (Zingaretti et al., 2013). Monitoring metabolite levels over time, to create a dynamic picture, demonstrated the ability of the tolerant potato clones to fine tune metabolite compositions to the degree of stress experienced by the plant and its capacity to deal with this stress condition (Vasquez-Robinet et al., 2008). Overall, the incorporation of metabolite profiling datasets augments the elucidation of the biochemical mechanisms underlying the physiological adaptations.

4. Experimental

4.1 Plant material and experimental design

Five potato genotypes differing in their sensitivity to water stress (Table 1) (Cabello et al., 2012; Carli et al., 2014; Ramírez et al., 2015a) were selected for their similar vegetative maturity (≤120 days) and population background from CIP breeding program. Four of the clones are part of the lowland tropic virus resistant (LTVR) population (Bonierbale et al., 2003) and the fifth clone is part of an ancient population bred for resistance to bacterial wilt (BW) (Table 1) (Mihovilovich et al., 2015).

The experiment was conducted under greenhouse conditions from September to December 2013, at CIP experimental station in Huancayo, Peru (3200 meter above sea level). Atmospheric temp. and humidity (HOBO U23 Pro v2 Temp./Relative Humidity Data Logger –U23-001, Onset Computer Corporation, Bourne, MA, USA) were recorded every 10 min using a mini data –logger (HOBO U12 Outdoor/Industrial Data Logger, Onset Computer Corporation, Bourne, MA, USA) (Table 2).

Tubers of the five genotypes were planted in plastic trays filled with 1:1 rinsed fine –textured sand and gravel and after 22 days later, thirty vigorous sprouts of each breeding clone were extracted (cut out from the tubers) and sown individually in 12 inches pots filled with 15kg of the same sand and gravel mixture. Pots were distributed following a split block design with three replicates of ten pots per genotype; half of them received a different water treatment. Plants grown from sprouts were watered by drip irrigation (4L/h) and all fertilisers were injected directly into the irrigation water. Fertilisation was performed with 5.18g l^{-1} potassium nitrate, 3.36 g l^{-1} ammonium nitrate, 2.68 g l^{-1} calcium triple superphosphate, 1.15g l^{-1} magnesium sulphate, 120g l^{-1} fetrilon

combi micronutrients, and 96g l^{-1} iron quelate. Pest control was carried out with the application of two insecticides Rovral (active ingredient: Iprodione; Bayer CropScience, Peru) and Beta baytroid (active ingredient: Beta cyfluthrin; Bayer CropScience, Peru) at 0.16 g l^{-1}.

All plants were watered every other day for 30 days before the water restriction (WR) treatment initiation. The WR treatment consisted of applying half of the amount of water supplied to the control treatment (CT). Irrigation frequency in WR was performed in an interval of around nine days depending on daily temp. (Table 2) which was repeated eight times over a total period of 80 days. The CT plants continued to be watered with the same frequency as the first 30 days.

4.2 Physiological and agronomical measurements

4.2.1 Total biomass, Harvest Index determination and Drought Tolerance Index

At harvest (80 days after water restriction initiation (DAWRI)), total fr. wt and dry wt were determined. The dry wt of each plant was measured, in triplicate or more, after drying all the components at 80°C for 3 days in a forced air oven. The harvest index (HI) was calculated as the ratio of DW tuber (g) to the total DW biomass (g). The drought tolerance index (DTI) was calculate according to DTI= ((yield under stress x yield under control)/mean yield under control) (Saravia et al., 2016).

4.2.2 Osmotic potential (OP)

Leaf osmotic potential was determined at 0, 15, 35 and 70 DAWRI in leaf discs of 5mm diameter, taken from the third fully extended leaf of at least three plants per clone and

treatment. Leaf discs were transferred into cryogenic tubes and frozen in liquid nitrogen for further analysis. The frozen leaves were incubated at 22°C for 30 min in a sealed C-52 chamber (Wescor Inc., Logan, UT, USA) prior to determination of the osmotic potential with a dew point microvoltmeter (HR-33T from the same company).

4.2.3 Relative water Content (RWC)

RWC was determined at 0, 15, 35 and 70 DAWRI by weighing the third leaflet from the youngest fully expanded leaf (third leaf from the apical part) (fr. wt) and placing it in a 4x3 inch Ziploc bag containing distilled water for 24 h. Excess water was removed by blotting each leaf in a paper towel prior to taking turgid weight (TW). Leaves were then dried in an oven at 90 ºC overnight and reweighed (dry wt). RWC was calculated according to RWC (%) = [(fr. wt–dry wt) / (TW–dry wt)] x 100 (Barrs et al., 1962). The RWC mean value was established from triplicate measurements of each clone per treatment.

4.2.4 Chlorophyll content index (CCI)

Chlorophyll content was determined at 0, 15, 25 and 70 DAWRI as a CCI value using a portable chlorophyll meter (Minolta SPAD-502, Konica Minolta, Sakai, Osaka, Japan). In each plant, three leaflets of the youngest fully expanded leaf (third node) were used to measure the transmittance of red (650 nm) and infrared (940 nm) radiation through the leaf. The average CCI of the three leaflets provided by the average option of the instrument was recorded as an individual CCI plant value. This was repeated in at least three plants of each clone per treatment and replication.

4.2.5 *Photosynthetic activity (A_{sat}) and stomatal conductance (g_s)*

Light-saturated net CO_2 assimilation rate (A_{sat}) and stomatal conductance (g_s) were measured at 15, 25 and 70 DAWRI on the youngest fully expanded leaves, in at least three plants of each clone per treatment and replication. A_{sat} and g_s were measured with a portable gas exchange system (LI-6400, Li–COR, Lincoln, USA). Leaf chamber conditions was set up at 385 ppm CO_2, 23.5 ºC (block temp.), a saturated photon flux density (1500 µmol m^{-2} s^{-1}) and ambient relative humidity.

4.3 *Metabolite analysis*

4.3.1 *Extraction of metabolites*

Leaf tissue samples at 30 DAWRI and leaf, tuber and root tissue samples at 70 DAWRI of both treatments were frozen in liquid nitrogen immediately after harvest and ground to powder. Samples, including quality controls (pool of all samples, QC), were weighed (10±0.5 mg) in plastic tubes and extracted as described previously (Nogueira et al., 2013). Aliquots of the polar and non-polar phase were immediately dried down after extraction.

4.3.2 *GC-MS analysis.*

An aliquot of the polar phase (200µl) was removed and internal standard ([D4]Succinic acid, 10µg) added before dry down. Dried samples were derivatised as previously described (Nogueira et al., 2013) and analysed by GC-MS based on literature (Enfissi et al., 2010), using a 10:1 split mode. Metabolites were identified with respect to an in-house library based on retention time, retention indices and mass spectrum (Nogueira et al., 2013) and quantified relatively to the internal standard.

4.3.3 LC-MS analysis

Each dried aliquot of the polar phase (700µl) was resuspended in methanol/water (1:1, 100µl) and internal standard (homogenistic acid, 5µg) added. Samples were filtered using syringe filter (nylon, 0.45µm) before analysis based on a published LC-MS method (Enfissi et al., 2010) with modification of solvents A (water and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid). The gradient was held at 100% A for 1 min, followed by a gradient up to 35% B until 18 min and to 95% B until 19 min. The gradient was then held at 95% B for 4 min and the column returned to the initial conditions within 1 min and equilibrated for 5 min. Data analysis was performed based on R package metaMS (Shahaf et al., 2013; Wehrens et al., 2015) with a retention time window match set to 0.5min.

4.3.4 Chromatographic analysis with UPLC

For analysis of carotenoids and chlorophylls, an aliquot of the non-polar phase (350µl) was dried, resuspended in ethyl acetate/acetonitrile (1:9) and analysed as previously described (Nogueira et al., 2013). Metabolites were identified through specific retention time and UV/visible light spectrum and quantified from dose-response curves (Fraser et al., 2000).

4.3.5 Data processing and statistical analysis

Principal component analysis (PCA) using a correlation matrix to standardise the variables was performed to identify components which contributed the greatest variance between genotypes. Statistical analysis was perform using JMP® Pro 12.0.1 (SAS Institute Inc, Cary, NC) and Simca P 13.0.3.0 (Umetrics, Sweden). Statistical analysis

was performed using the computing environment R v 3.2.4 (The R Foundation for Statistical Computing, 2016). Changes in identified metabolites between WR and CT samples were calculated as average ratios. Their significance was established through pairwise comparison with Student's *t* test for the metabolic data and Fisher's least significant difference (LSD) for the physiological data ($P < 0.05$). Results for leaves were displayed in a heat map using Excel 2013 (Microsoft Office, USA). Metabolite changes in tuber were overlaid with biochemical pathways constructed specifically with BioSynLab software (wwww.biosynlab.com).

Acknowledgements

The authors would like to thank Mariela Aponte and Chris Gerrish for their excellent technical assistance. This work was supported by the CGIAR Research Program on Roots, Tubers and Bananas (RTB).

Tables

Table 1: Genotypes analysed in the study

Population	CIP Number	Variety name (first country of release)	Female parent	Male parent	Drought sensitivity
LTVR	392797.22	UNICA (Peru)	387521.3	Aphrodite	Tolerant
LTVR	394611.112		780280=(PW-88-6203)	676008=(I-1039)	Susceptible
LTVR	390637.1		PW-31	385305.1=(XY.9)	Medium–tolerant
LTVR	397077.16	SARNAV (Uzbekistan)	392025.7=(LR93.221)	392820.1=(C93.154)	Tolerant
BW	395448.1		393617.1=(TXY.11)	BWH-87.344R	Susceptible

Table 2: Monthly averages of maximum and minimum temp. and relative humidity measured from September to December 2013.

	September	October	November	December
Max. temp. (ºC)	28.3 ± 0.6	26.6 ± 0.4	26.5 ± 0.4	24.4 ± 0.5
Min. temp. (ºC)	5.6 ±0.5	7.9 ± 0.3	7.5 ± 0.3	8.8 ± 0.2
Average temp. (ºC)	14.7 ± 0.3	14.9 ± 0.2	15.1 ± 0.2	14.6 ± 0.2
Max. rel. humidity (%)	90.8 ± 1.1	94.1 ± 0.6	95.3 ± 0.5	97.8 ± 0.4
Min. rel. humidity (%)	19.9 ± 1.3	27.1 ± 1.2	26.3 ± 1.4	36.2 ± 1.3

Table 3: Mean ± s.d. (n=3) of physiological parameters under control (CT) and water restriction (WR) treatments of five breeding clones. Fischer's least significant difference (LSD) values at $p<0.05$ were used to compare between treatments by clone (significant difference are indicated in bold) and between clones by treatment (different letters indicate statistically significant difference). DAWRI= Days after water restriction initiation, RWC= Relative Water Content (%), OP= Osmotic potential (bar), A_{sat}= Photosynthetic activity (µmol.m^{-2}.s^{-1}), g_s= Stomatal conductance (µmol.m^{-2}.s^{-1})

		CCI				RWC				A_{sat}		
Clones	**Treatment**	**0DAWRI**	**15DAWRI**	**25DAWRI**	**70DAWRI**	**0DAWRI**	**15DAWRI**	**35DAWRI**	**70DAWRI**	**15DAWRI**	**25DAWRI**	**70DAWRI**
CIP395448.1	**CT**	**42.5** ^{a} ± 2.8	**49.0** ^{a} ± 3.1	**50.2** ^{a} ± 3.6	**50.6** ^{a} ± 3.2	91.7 ^{a} ± 6.6	88.2 ^{a} ± 5.8	**92.5** ^{a} ± 1.8	**90.8** ^{a} ± 1.9	10.4 ^{a} ± 4.9	9.6 ^{a} ± 5.7	9.6 ^{a} ± 2.2
	WR	**45.7** ^{a} ± 3.2	**52.7** ^{a} ± 3.8	**55.2** ^{a} ± 4.9	**54.9** ^{a} ± 8.6	92.9 ^{a} ± 4.7	88.8 ^{a} ± 2.1	**81.2** ^{a} ± 9.1	**69.1** ^{a} ± 13.7	5.4 ^{a} ± 5.3	9.6 ^{a} ± 2.9	7.2 ^{a} ± 3.7
CIP394611.112	**CT**	41.0 ^{ab} ± 2.9	48.4 ^{a} ± 1.8	**48.7** ^{a} ± 1.8	**48.3** ^{a} ± 2.5	92.4 ^{a} ± 6.9	89.1 ^{a} ± 4.9	**92.3** ^{a} ± 2.7	**91.8** ^{a} ± 2.5	15.3 ^{a} ± 1.2	9.8 ^{a} ± 7.5	4.4 ^{b} ± 3.9
	WR	42.7 ^{b} ± 3.1	49.6 ^{b} ± 1.3	**53.2** ^{ab} ± 2.6	**55.1** ^{a} ± 2.1	92.0 ^{a} ± 4.4	85.5 ^{ab} ± 5.4	**82.2** ^{a} ± 7.9	**60.6** ^{b} ± 5.8	5.9 ^{a} ± 4.6	9.8 ^{a} ± 7.7	7.1 ^{a} ± 5.6
CIP392797.22	**CT**	37.7 ^{cd} ± 2.5	45.2 ^{a} ± 1.8	46.3 ^{a} ± 1.9	**43.3** ^{b} ± 2.7	92.5 ^{a} ± 7.8	85.8 ^{a} ± 5.7	**92.3** ^{a} ± 3.7	**91.3** ^{a} ± 2.8	6.8 ^{a} ± 4.6	10.5 ^{a} ± 10.1	6.0 ^{ab} ± 3.3
	WR	39.4 ^{c} ± 2.9	45.5 ^{c} ± 1.4	50.1 ^{b} ± 2.8	**47.9** ^{b} ± 2.0	92.2 ^{a} ± 4.8	81.2 ^{b} ± 8.6	**78.2** ^{a} ± 8.4	**69.3** ^{a} ± 3.4	8.0 ^{a} ± 6.2	9.7 ^{a} ± 6.1	7.2 ^{a} ± 3.9
CIP397077.16	**CT**	**39.1** ^{bc} ± 1.3	43.2 ^{bc} ± 1.7	**40.1** ^{b} ± 1.1	**39.7** ^{bc} ± 2.3	95.8 ^{a} ± 2.8	85.5 ^{a} ± 3.0	**91.2** ^{a} ± 6.0	**93.3** ^{a} ± 1.3	8.5 ^{a} ± 4.0	8.4 ^{a} ± 4.5	7.6 ^{ab} ± 4.6
	WR	**41.5** ^{b} ± 1.4	43.8 ^{cd} ± 0.9	**45.1** ^{c} ± 3.3	**34.6** ^{c} ± 2.7	94.4 ^{a} ± 4.1	85.6 ^{ab} ± 4.4	**81.7** ^{a} ± 10.1	**60.4** ^{b} ± 9.7	11.1 ^{a} ± 8.6	9.9 ^{a} ± 5.0	8.9 ^{a} ± 4.5
CIP390637.1	**CT**	36.9 ^{d} ± 2.3	40.4 ^{c} ± 2.5	**40.1** ^{b} ± 2.4	38.8 ^{c} ± 3.3	92.7 ^{a} ± 5.0	84.3 ^{a} ± 5.5	**93.7** ^{a} ± 1.0	**94.1** ^{a} ± 1.9	13.5 ^{a} ± 7.8	7.6 ^{a} ± 3.7	10.2 ^{a} ± 3.0
	WR	38.8 ^{c} ± 3.2	42.2 ^{d} ± 3.0	**45.0** ^{c} ± 4.3	36.5 ^{c} ± 4.3	93.2 ^{a} ± 6.7	82.5 ^{b} ± 6.6	**79.9** ^{a} ± 7.4	**72.3** ^{a} ± 9.6	10.9 ^{a} ± 8.8	9.8 ^{a} ± 6.5	9.0 ^{a} ± 3.6
LSD values		1.93	2.83	3.93	4.23	4.51	4.83	7.36	8.31	22.87	5.02	5.21

		OP				Total Biomass		Harvest Index		g_s		
Clones	**Treatment**	**0DAWRI**	**15DAWRI**	**35DAWRI**	**70DAWRI**	**fr. wt**	**dry wt**	**fr. wt**	**dry wt**	**15DAWRI**	**25DAWRI**	**70DAWRI**
CIP395448.1	**CT**	-24.2 ^{a} ± 2.2	**-24.0** ^{a} ± 3.8	-21.7 ^{a} ± 3.5	-26.0 ^{a} ± 3.0	**750.5** ^{c} ± 482.2	**84.0** ^{bc} ± 54.4	**31.9** ^{c} ± 22.2	**29.8** ^{c} ± 20.1	0.025 ^{c} ± 0.01	0.020 ^{a} ± 0.02	0.037 ^{a} ± 0.02
	WR	-21.5 ^{a} ± 2.7	**-20.7** ^{a} ± 4.5	-21.2 ^{b} ± 4.9	-27.4 ^{a} ± 4.6	**207.0** ^{a} ± 106.3	**24.0** ^{a} ± 10.3	**40.5** ^{d} ± 25.6	**44.6** ^{b} ± 30.6	0.017 ^{a} ± 0.03	0.036 ^{a} ± 0.03	0.019 ^{a} ± 0.01
CIP394611.112	**CT**	-21.5 ^{a} ± 3.8	-19.3 ^{c} ± 3.7	**-16.9** ^{b} ± 3.7	**-21.3** ^{b} ± 4.8	**1043.0** ^{a} ± 817.7	**101.9** ^{a} ± 66.1	**43.2** ^{b} ± 16.8	**51.5** ^{b} ± 15.2	0.063 ^{bc} ± 0.02	0.032 ^{a} ± 0.04	0.035 ^{a} ± 0.03
	WR	-20.4 ^{a} ± 2.9	-18.4 ^{a} ± 3.2	**-20.1** ^{b} ± 4.2	**-24.9** ^{a} ± 3.3	**247.6** ^{a} ± 87.9	**29.2** ^{a} ± 10.8	**61.4** ^{c} ± 25.8	**69.4** ^{a} ± 18.9	0.020 ^{a} ± 0.02	0.017 ^{a} ± 0.02	0.012 ^{a} ± 0.01
CIP392797.22	**CT**	-21.5 ^{a} ± 3.8	-20.7 ^{bc} ± 2.1	-20.3 ^{a} ± 2.6	-26.0 ^{a} ± 2.8	**1025.2** ^{ab} ± 721.7	**100.7** ^{ab} ± 61.8	**49.0** ^{b} ± 15.3	**58.2** ^{b} ± 12.7	0.021 ^{c} ± 0.03	0.022 ^{a} ± 0.04	0.016 ^{a} ± 0.02
	WR	-21.9 ^{a} ± 4.7	-19.5 ^{a} ± 3.1	-21.1 ^{b} ± 3.9	-27.3 ^{a} ± 4.2	**255.9** ^{a} ± 104.7	**29.7** ^{a} ± 9.5	**64.3** ^{bc} ± 23.2	**71.2** ^{a} ± 17.3	0.023 ^{a} ± 0.02	0.016 ^{a} ± 0.02	0.018 ^{a} ± 0.02
CIP397077.16	**CT**	-22.4 ^{a} ± 2.6	**-23.1** ^{ab} ± 2.2	**-22.3** ^{a} ± 4.4	-27.2 ^{a} ± 3.4	**799.1** ^{c} ± 632.6	**76.6** ^{c} ± 46.3	65.7 ^{a} ± 21.9	73.9 ^{a} ± 17.3	0.120 ^{ab} ± 0.13	0.021 ^{a} ± 0.03	0.014 ^{a} ± 0.01
	WR	-19.8 ^{a} ± 3.0	**-18.3** ^{a} ± 3.5	**-24.7** ^{b} ± 3.3	-27.5 ^{a} ± 3.4	**229.4** ^{a} ± 89.6	**28.7** ^{a} ± 10.7	71.6 ^{ab} ± 22.4	78.7 ^{a} ± 15.6	0.026 ^{a} ± 0.03	0.040 ^{a} ± 0.04	0.009 ^{a} ± 0.01
CIP390637.1	**CT**	-25.0 ^{a} ± 3.0	-20.8 ^{bc} ± 2.4	-20.2 ^{a} ± 3.4	-25.2 ^{a} ± 2.9	**832.1** ^{bc} ± 613.0	**70.8** ^{c} ± 43.7	**61.0** ^{a} ± 18.4	68.8 ^{a} ± 17.1	**0.154** ^{a} ± 0.22	0.018 ^{a} ± 0.02	0.049 ^{a} ± 0.03
	WR	-23.7 ^{a} ± 4.2	-19.9 ^{a} ± 1.3	-21.6 ^{b} ± 4.1	-24.7 ^{a} ± 4.0	**227.8** ^{a} ± 99.6	**29.1** ^{a} ± 17.7	**72.5** ^{a} ± 22.2	74.4 ^{a} ± 18.9	**0.028** ^{a} ± 0.04	0.034 ^{a} ± 0.03	**0.031** ^{a} ± 0.04
LSD values		4.06	2.88	2.15	3.36	206.92	17.1	7.73	9.61	0.072	0.024	0.037

Figure Legends

Figure 1. PCA analysis of leaf, tuber and root at 30 and 70 days of the water restriction and control treatment. Score plot was based on the metabolite composition of leaf (A,B), tuber (C) and root (D) of five potato genotypes at 30 (A) and 70 (B-D) days under control treatment (CT, grey) and water restriction (WR, white). Metabolite variables were obtained from untargeted analysis of the polar extracts.

Figure 2. Heatmap displaying significant differences of metabolite levels in leaf (30 and 70 days) of five potato genotypes under water restriction compared to control treatment conditions. Metabolites were determined from targeted analysis of polar and non-polar extracts. Fold changes are indicated as decrease (red), no change (grey) and increase (blue). Fold changes higher than 2.5 are indicated as blue with black dots.

Figure 3. Pathway display of significant changes in metabolite levels in tubers of five potato genotypes under water restriction compared to control treatment conditions. Metabolites were determined from targeted analysis of polar extracts. Fold changes are indicated as decrease (red), no change (grey) and increase (blue). Metabolites not detected in the targeted analysis are indicated in white.

Supplementary data

Supplementary Fig. 1. Drought tolerant index (DTI) of tuber yield (A) of five genotypes and PCA displayed as biplot (B) of control treatment (CT, grey) and water restriction (WR, white) treatments including scores (genotypes) and loadings (physiological measurements). Physiological traits were measured over the whole duration of the experiment.

Supplementary Fig. 2. PCA to assess metabolite composition of leaf of five potato genotypes at 30 (A,B) and 70 (C,D) days under control treatment (CT; A,C) and water restriction (WR; B,D) treatment. Metabolites variables were obtained from untargeted analysis of polar extracts.

Supplementary File 1. Effect of treatment, genotypes, and their interaction on physiological parameters measured in 5 breeding clones at different times points Values are ANOVA Mean square and P -values are shown.

Supplementary File 2. Data of untargeted analysis of leaf, root and tuber material under control treatment (CT) and water restriction (WR) conditions at 30 and 70 DAWRI.

Supplementary File 3. Average ratios and *P*-Values of targeted analysis under water restriction (WR) conditions compared to the control (CT).

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Figure 1

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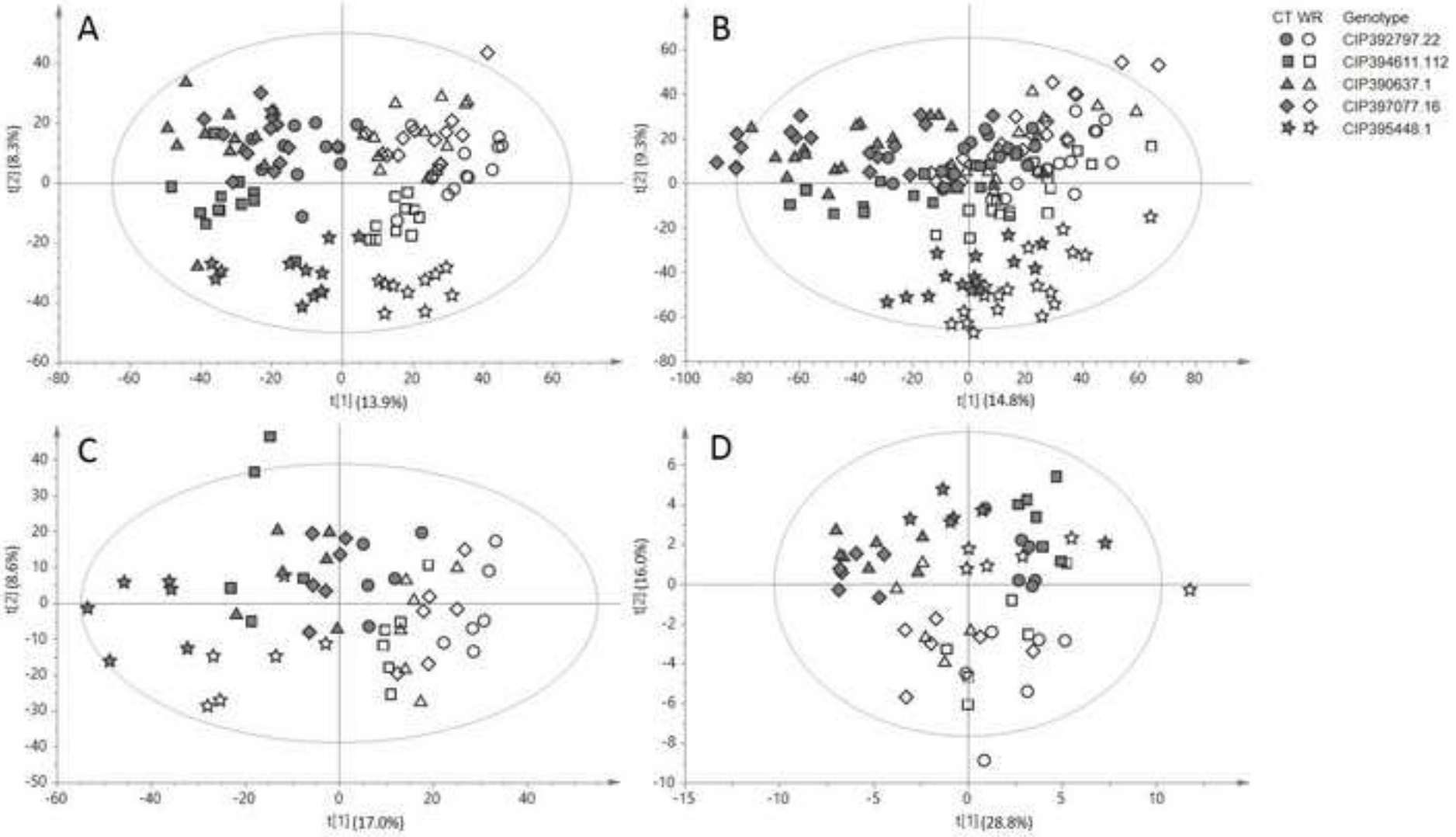

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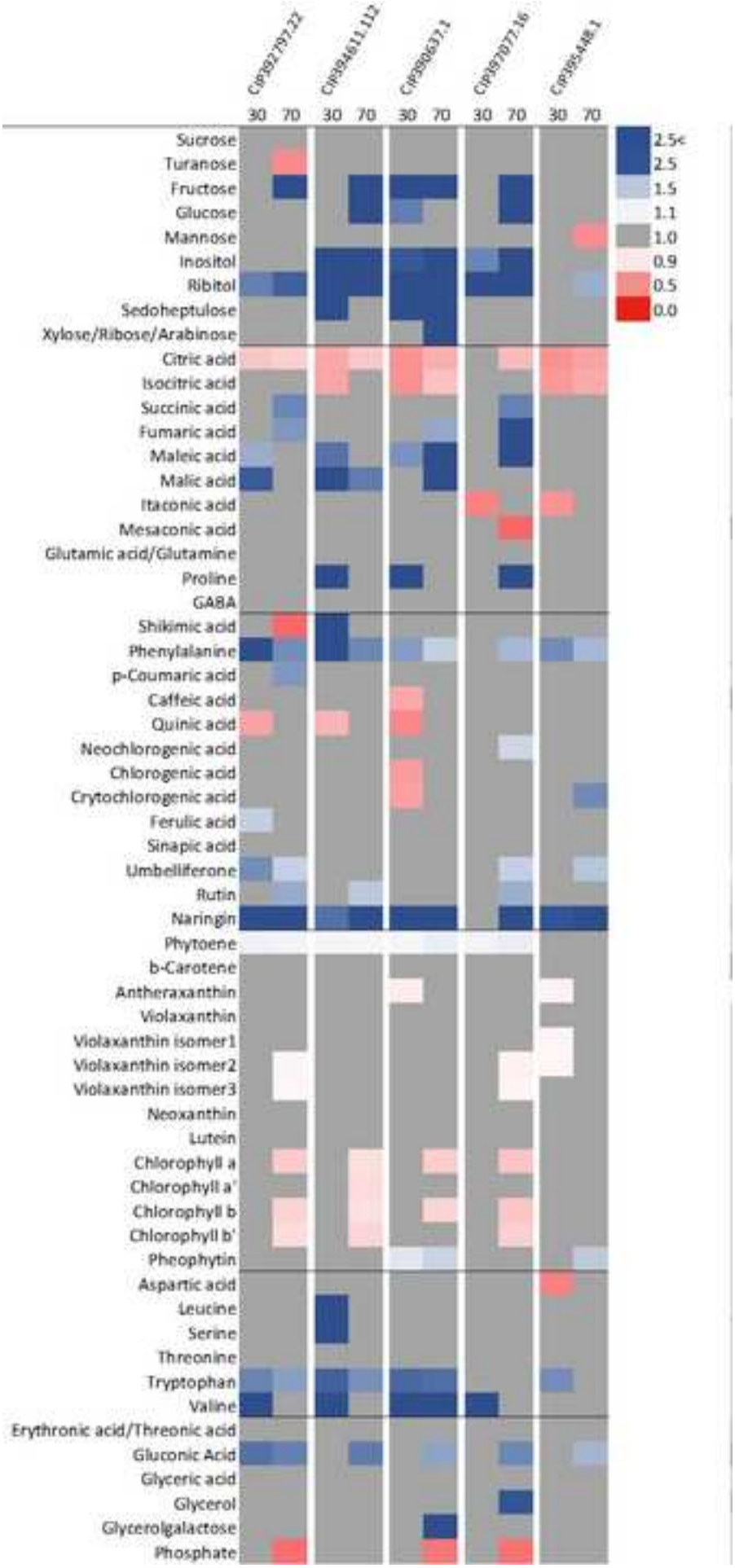

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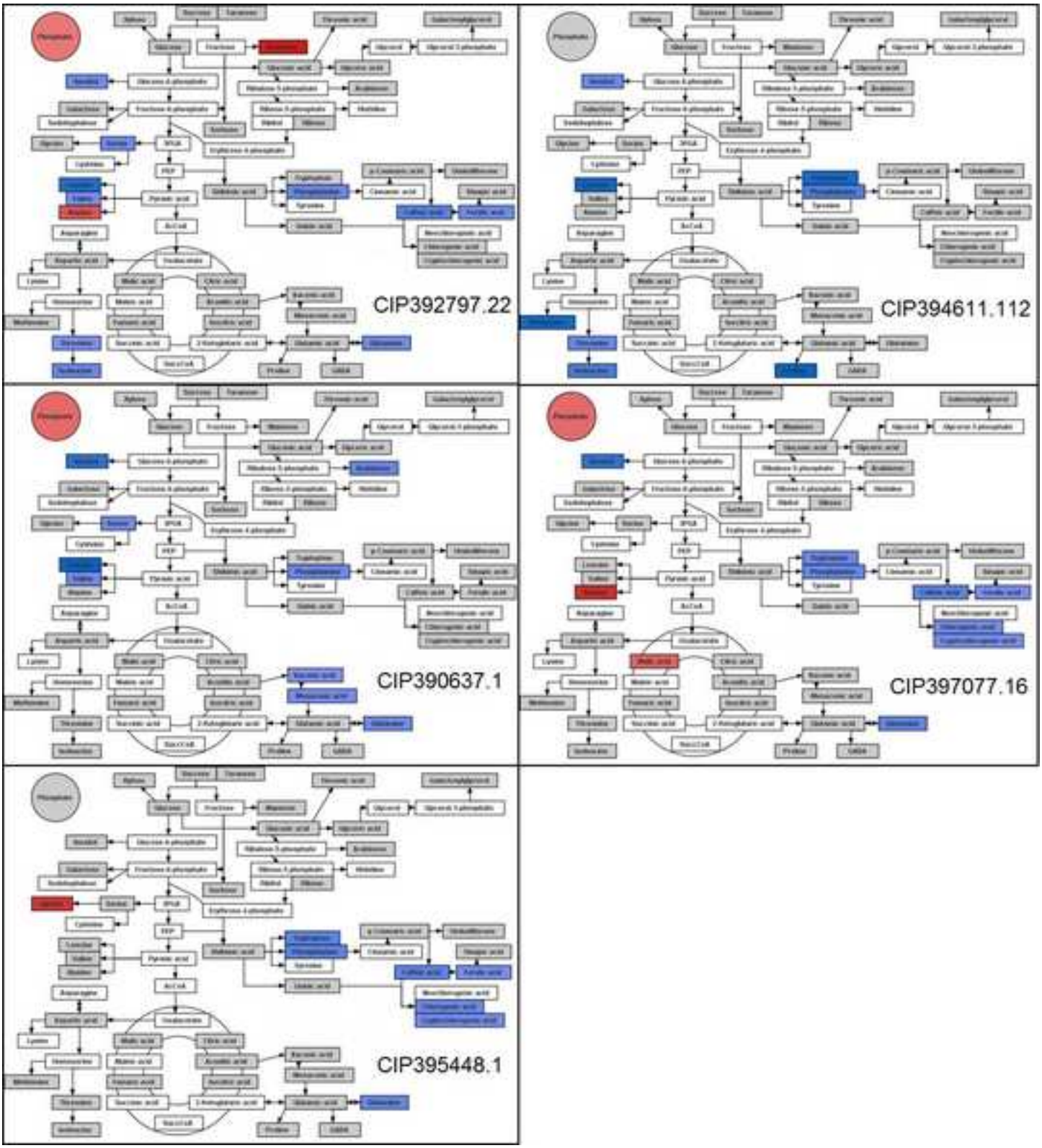

Supplementary Information

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Supplementary Information

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Supplementary Information

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