Moderate to severe water limitation differentially affects the phenome and ionome of Arabidopsis

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Abstract. Food security is currently one of the major challenges that we are facing as a species. Understanding plant responses and adaptations to limited water availability is key to maintain or improve crop yield, and this is even more critical considering the different projections of climate change. In this work, we combined two high-throughput ‘omic’ platforms (‘phenomics’ and ‘ionomics’) to begin dissecting time-dependent effects of water limitation in Arabidopsis leaves and ultimately seed yield. As proof of concept, we acquired high-resolution images with visible, fluorescence, and near infrared cameras and used commercial and open source algorithms to extract the information contained in those images. At a defined point, samples were also taken for elemental profiling. Our results show that growth, biomass and photosynthetic efficiency were affected mostly under severe water limitation regimes and these differences were exacerbated at later developmental stages. The elemental composition and seed yield, however, changed across the different water regimes tested and these changes included under- and over- accumulation of elements compared with well-watered plants. Our results demonstrate that the combination of phenotyping techniques can be successfully used to identify specific bottlenecks during plant development that could compromise biomass, yield, and the nutritional quality of plants.

Additional keywords: Arabidopsis thaliana, drought stress, high-throughput plant phenotyping, ionomics, phenomics.

Introduction

Water availability is one of the major factors limiting plant growth and yield worldwide and global climate change is expected to compromise water resources around the globe even further (Chaves et al. 2003). At the same time, world population is growing and the demand for food is also expected to rise over the next decades. Thus, the challenge ahead of us is to develop crop varieties able to endure prolonged periods of stress without experiencing significant losses in nutritional quality or yield. Significant advances have been made at the physiological and molecular level to understand how some plant species are able to thrive in places where water is scarce (Yang et al. 2015). In addition, plant responses to water limitation have been documented extensively in model and crop plants using growth chambers, greenhouses, field conditions and even different growth media (e.g. agar, soilless systems and soil with different degrees of water saturation). Morphological and physiological changes that occur during water limitation stress include decrease in transpiration and photosynthesis, and reduced biomass (Boyer 1970; Tardieu et al. 1999; Farooq et al. 2009). Metabolic changes that have also been reported in response to water limitation include the reduction of net carbon assimilation rate due to stomatal closure and low CO\textsubscript{2} diffusion, leading to a downregulation of the photosynthetic machinery (reviewed by Farquhar and Sharkey 1982; McDowell et al. 2008 and Farooq et al. 2009). For many crop species, limited water availability impacts yield and the severity of yield loss depends on the level and duration of the stress (Farooq et al. 2009). Since yield integrates many phenotypical and physiological processes in a complex way, it is often difficult to predict what type of changes and adaptations during the life cycle of the plant ultimately will led to the observed yield penalty when plants experience water limitation stress. All these studies have provided valuable insights into the different responses and adaptations that plants have evolved when water is limiting. These studies have also made clear that the timing of treatment, the plant developmental stage, and the severity of the treatment are critical to define the type and magnitude of the plant response (for a detailed review about physiological and molecular responses to drought see Claeys and Inzé 2013).

Over the last decade, significant technological advances have made possible the tracking of plant growth and development in a high-throughput and automated manner. This approach, termed high-throughput plant phenotyping...
(HTPP or ‘phenomics’), allows the acquisition and quantification of phenotypes from hundreds and even thousands of plants in a short period of time. HTPP platforms typically use high-resolution cameras to capture images in the visible, fluorescence, and infrared ranges to quantify plant size, architecture, colour, in planta chlorophyll content, in planta water content, and leaf temperature among other readouts of interest (Fahlgren et al. 2015a). Benefits from HTPP approaches include the fact that these methods are fast, non-invasive, unbiased and accurate. In addition, if documentation of those HTPP experiments is done properly, the captured images can be reanalysed and additional information can be extracted if novel algorithms or additional questions emerge after the experiments have been completed (Fahlgren et al. 2015a; Junker et al. 2015). Additional high-throughput techniques, often called –’omic’ approaches, have also been established to identify and quantify metabolites (‘metabolomics’), the elemental composition or ions (‘ionomics’), gene expression (‘transcriptomics’) and protein abundance (‘proteomics’) in biological samples. In principle, these techniques can be used independently of each other towards a full of understanding of a biological system; however, data integration and interpretation of separate high-throughput approaches has proven to be challenging. This is particularly true when these techniques are applied in different laboratories, under slightly different experimental settings, thus altering the timing and magnitude of the plant response, which complicates later the ability to integrate -omics data seamlessly. To address this issue, we have begun the systematic combination of high-throughput technologies to first identify critical points during plant development where additional -omic techniques can be brought in to increase the depth of analysis in a more meaningful way.

Plants affected by water limitation need to undergo a period of osmotic adjustment and this process is necessary to maintain water retention and turgor (Wang et al. 2013), thus allowing the cells to tolerate better the drought stress. This is possible in part by a variety of modifications that include changes in ion uptake, distribution and growth arrest. ‘Ionomics’, or the study of the elemental composition of an organism or tissue, has been successful at identifying genes responsible for controlling the accumulation of one or a group of elements in different organisms (Lahner et al. 2003; Eide et al. 2005). One area that has received much less attention, despite its physiological relevance, is the effect of water limitation on the plant ‘ionome’. Here, as proof-of-concept, we first used HTPP to document the ‘phenome’ of Arabidopsis grown under different water availability regimes. From these data, we selected a developmental stage where an additional high-throughput technology (‘ionomics’) was used to monitor the elemental composition of plants grown under different water regimes. Our data support the idea that the sequential use of high-throughput technologies may be beneficial to increase the analytical depth at critical points during plant development. Future work could definitely be escalated to include as many techniques and points as the researchers consider necessary to understand a biological system better, but the systematic use of these technologies will likely help with the analysis and data interpretation, which is still a limiting step of high-throughput technologies.

Materials and methods

Plant growth

Arabidopsis thaliana (L. Heynh.) (Col-0, CS-60000) seeds were obtained from the Arabidopsis Biological Resource Centre (The Ohio State University, Columbus, OH, USA). Seeds were surface sterilised sequentially with 70% ethanol, 50% bleach, 0.05% Tween 20 and finally rinsed with sterile water before being plated on MS media (Murashige and Skoog 1962) supplemented with 3% sucrose. Seeds were vernalised for 3 days at 4°C before being transferred to an environment controlled chamber (Conviron) at 22 ± 1°C, 65 ± 5% RH, and 160–200 μmol m⁻² s⁻¹ light intensity on a short day photoperiod (10 h day, 14 h night). After true leaves formed (12 days after sowing), the most vigorous seedlings were transferred into 85 × 73 mm Quick Pot 15 RW trays (HerkulPlast Kubern GmbH) containing Arabidopsis plant growing media (Lehle Seeds). Blue mesh (Ktritrich Corporation) was placed on top of the soil mixture to prevent algae growth and to improve the object segmentation during image analysis (Fig. 1). Plants were grown to maturity under these conditions until the start of the water limitation treatments (16 days after germination).

Water treatments

Quick Pot 15 RW trays were filled with dry Arabidopsis plant growing media and their weight was recorded. Measured amounts of water were added to the dry soil and allowed to absorb for 1 h until soil reached full water saturation (100% full water capacity, FC), after which tray weights were once again recorded. After seedling establishment (~16 days after germination), soil was allowed to reach four different levels of water saturation: 100 (control), 50, 25 and 12.5%. The weight of the trays was checked daily and water was uniformly added to all wells until the target weight was reached.

Soil water potential meter

Soil water potential (Ψ MPa) was measured with a soil water potential meter (WP4C, Decagon). All measurements were conducted at the same time of the day. Soil samples were taken from five wells within the Quick Pot 15 RW tray, then placed in a round sample cup (4 cm in diameter and 1 cm tall) and set in the sample drawer. This instrument measured water potential by determining the RH of the air above of the sample in a closed chamber. Once the sample reached equilibrium with the vapour in the sealed chamber, the instrument calculated the RH by using the chilled mirror method. At the dew point, the meter measured both mirror and sample temperature with a 0.001°C accuracy delivering water potential readings with accuracy within the −0.1 MPa to −300 MPa range.

Image acquisition

Images of Arabidopsis plants were captured every 2 days; from seedling establishment to a full vegetative growth (developmental stage 6.1 as defined by Boyes et al. 2001) using a LemnaTec Scanalyzer HTS system controlled using the LemnaControl software. This automated imaging system is equipped with a robotic arm that holds three high-resolution cameras that allows to image the top view on the visible (VIS), fluorescence (FLUO) and near infrared (NIR) spectra.
The system also has a barcode reader placed in a cabinet with optimal light conditions (Fig. 1). Images were captured during a specific time window each day (3.5 h after onset of daylight ± 30 min). VIS images were taken using a piA2400–17 gc CCD camera (Basler) with a resolution of 2454 × 2056 pixels. A scA1600–14 gc CCD camera (Basler) equipped with a resolution of 1624 × 1234 pixels was used for the acquisition of the FLUO images. NIR images were taken with a Goldeye GIGE P-008 SWIR camera (Allied Vision Technologies) equipped with a resolution of 320 × 256 pixels and with spectral sensitivity between 900 and 1700 nm. The LemnaControl software allowed for detailed configuration of the pots, trays, barcode positions and camera settings (zoom focus, aperture and shutter speed).

Image analysis

Images of A. thaliana plants (4320 images = 30 biological replicates × four treatments × 12 time points × three cameras) were analysed by using the LemnaGrid software. Analysis of the images acquired with the VIS camera was done as previously described (Arvidsson et al. 2011). Once all the rosette leaves were identified, multiple phenotypic parameters were calculated for each plant including: projected leaf area (cm²), convex hull area (cm²), caliper length (mm), and compactness (the ratio of projected leaf area to convex hull area, a measure of the ‘bushiness’ of the plant). The LemnaGrid software was also used to colour classify the images acquired with the VIS camera and, based on this colour classification, calculate the relative leaf area with normal green colour versus the area with detectable yellow colour. A similar colour classification approach was followed for the grey-scale images acquired with the NIR camera (high water corresponds to darker tones while low water corresponds to lighter colours). The complete greyscale (pixels range in value from 0 to 255, where 0 is black and 255 is white) was divided into three bins with centres equally distributed over the whole range (histogram with three bins), and the software used to calculate the relative leaf area with low, medium and high water content. Images acquired with the FLUO camera were analysed using the LemnaGrid software, in which the complete red scale was divided into 4 bins with centres equally distributed over the entire range and the software was programmed to calculate the relative leaf area with zero, low, medium and high chlorophyll fluorescence. All raw images, analysed images and calculations were saved into a PostgreSQL database (LemnaDB) for storage. Quantitative data obtained from the images was exported as CSV files and analysed in Excel (Microsoft Corporation).
Leaf number analysis
To determine leaf number, VIS images were exported from the LemmaDB database and processed by using a desktop computer running the Ubuntu system with an algorithm developed in-house (available upon request). Briefly, the analysis included five steps: histogram equalisation, plant object segmentation, mask refinement, Euclidean distance map calculation, and marker based watershed transformation. Leaf number estimation was stopped at 29 days due to the overlapping nature of Arabidopsis leaves, which severely decreases the accuracy of this automated pipeline, particularly when plants have more than four levels of overlapping leaves.

Photosynthetic efficiency
Photosynthetic efficiency was measured using a MultispeQ instrument developed by Dr David Kramer Laboratory (Michigan State University). This instrument combines the functionality of a handheld fluorometer, a chlorophyll meter and a benchtop spectrometer. Non-destructive measurements were taken every other day from 15 plants chosen randomly at the same time of day. Data was visualised in an Android tablet (Samsung Galaxy Tab 4) and analysed in the PhotosynQ web portal (www.photosynq.org, accessed 2 September) (documentation for the MultispeQ including concepts and tutorials can also be found here). Photosynthetic efficiency was calculated by measuring chlorophyll fluorescence at different light conditions as described in Baker et al. (2007). In addition, the following readouts were determined: photosynthetic efficiency of PSII (ΦII), linear electron flow (LEF), non-photochemical quenching (NPQ), photon flux (vH+), conductivity of thylakoid membrane to protons (gH+), electrochromic shift (ECSII), and greenness (SPAD) of the leaves.

Seed yield
Seed number was calculated as follows: the weight of 100 seeds per treatment was determined multiple times and the average was used to determine the total number of seeds per treatment.

Elemental analysis
The elemental composition of plant samples was determined using inductively coupled plasma optical emission spectrometry (ICP-OES) as previously described (Mendoza-Cózatl et al. 2014). Briefly, leaves or seeds from plants exposed to different water capacity regimes (100, 50, 25 and 12.5%) were harvested and dried for 6 days in an oven at 60°C; then 20–40 mg of DW were re-suspended with 1 mL of nitric acid (HNO3, trace metal grade) and boiled at 90°C in a water bath for a total of 30 min (3 x 10 min intervals) to ensure a complete digestion. Samples were further diluted with ICP-grade water to 10 mL and the concentration of macronutrients (Ca, K, Mg and Na) and micronutrients (Cu, Fe, Mn and Zn) were determined by ICP-OES (Optima ICP-OES 8000 Spectrometer, Perkin Elmer). For this analysis, 15 biological replicates for each condition were used.

Statistical analysis
For statistical evaluation of the experiments, the PROC GLM procedure of the software SAS 9.4 (SAS Institute) was used. The variation in projected leaf area, caliper length, compactness, convex hull area, colour classification, relative chlorophyll fluorescence, relative water content, soil water potential, linear electron flow, leaf number and seed yield were analysed using ANOVA, General linear model analysis (GLM) and subsequent post-hoc analysis least significant difference (L.S.D.) (Fisher’s least significant difference range test) at α = 0.05. Data presented are means ± s.e.

Results
Optimisation of plant growth for high-throughput phenotyping and ionomic assays
The first step in our studies was to optimise the growth conditions, image acquisition, image analysis and ionomic analyses of A. thaliana plants grown under various regimes of water availability. As illustrated in Fig. 1, seeds were germinated on MS plates and incubated under environmentally controlled conditions. Once robust and healthy seedlings were obtained (12 days after sowing), they were transferred into trays containing soil at 100% full water capacity (FC). After seedling establishment, trays were divided into four water regimes: 100, 50, 25 and 12.5% FC and plants were maintained at that level of water saturation until they reached maturity. In all cases, humidity, light and temperature were kept at optimal conditions. Throughout the experiment, visible (VIS, also referred to as RGB), fluorescence (FLUO) and near infrared (NIR) images of plants were acquired every other day by using a multi-camera digital imaging system (Scanalyzer HTS, Fig. 2). The target weight of the trays was checked manually on a daily basis and soil moisture was measured using a water potential meter. Photosynthetic efficiency of the plants was determined with a MultispeQ instrument once leaves were large enough for these non-destructive measurements. Leaves and seed samples were taken 29 days after germination to assess changes in the elemental composition (ionome) of plants due to the different water availability regimes as indicated in Fig. 1. Finally, seeds were collected and counted to determine yield.

Water status and phenotypic variation
To ensure that plants grown under 50, 25 and 12.5% FC were under true water limitation conditions, compared with plants grown on 100% FC, soil moisture measurements were performed by using a water potential meter. Fig. 3a shows that water limitation regimes ranged from –0.5 MPa (50% FC) to −1.6 MPa (12.5% FC) and these ranges have previously used in moderate to severe water deficit experiments (Bhaskara et al. 2015; Durand et al. 2016). We noted that despite the fact that biomass was clearly affected under the two most severe water limitation regimes (12.5 and 25% FC), no sign of chlorosis or necrosis was observed in any of the applied water treatments (Fig. 3b). Fig. 4 shows representative images acquired with the VIS, FLUO, and NIR cameras of the HTTP system. Also included in this figure are the images obtained after background removal (image segmentation) and analysis using the commercial software LemmaGrid.
Water limitation effect on plant size, growth, and development

Abiotic stresses often have variable and gradual effects depending on the specific developmental stage of the plant. Therefore, it is highly valuable to employ non-destructive automated phenotyping methods to fully assess the effects of water limitation on the growth and development throughout the entire plant life cycle. Fig. 5 shows the impact of water limitation conditions on four readouts related to plant size and architecture calculated after VIS images were analysed: (1) projected leaf area, (2) convex hull area, (3) caliper length (rosette diameter) and (4) compactness, which is a measurement of the ‘bushiness’ of the rosette. As illustrated in Fig. 5a–d, Arabidopsis plants grown under moderate water limitation (50% FC) displayed no penalty in the vegetative growth compared with 100% FC. However, plants grown at lower water saturation (12.5 and 25% FC) display significant changes. We noted that most of these changes were significant only after day 27, which is 10 days after the water limitation treatment started (Fig. 5a–c).

Colour classification results showed no presence of yellow colour, indicative of loss of chlorophyll or chlorosis, in the images from plants grown under the various water regimes (data not shown).

Notably, the analysis of top view images yielded no differences in projected leaf area between plants grown at 50% FC compared with control plants (100% FC); however, visual inspection of the images indicated that plants grown between 50–12.5% FC had fewer but larger leaves compared with control plants. To determine whether water limitation had an impact on leaf number, we developed an algorithm (available upon request) to count the total number of leaves per plant after processing the VIS images. The analysis showed that 29 days after germination, and 2 weeks of water limitation regimes, plants in all treatments had significantly fewer leaves compared with control plants (Fig. 6).

Water limitation effect on leaf relative chlorophyll fluorescence

Chlorophyll fluorescence is used as an indicator to determine if a plant is tolerant to a particular abiotic stress. The FLUO camera of the HTPP platform and the associated software gave us the ability to measure relative chlorophyll fluorescence of Arabidopsis plants growing under various water regimes. Our results showed significant differences across treatments and in all of the fluorescence levels including the ‘no’, ‘low’, ‘medium’ ($P < 0.0001$) and ‘high’ fluorescence ($P < 0.0003$) categories (Fig. 7). Normal and severe water limitation conditions showed the highest area of zero fluorescence (labelled as ‘no’ relative chlorophyll fluorescence) and these were significantly different from the moderate soil water limitation regimes, including 25 and 50% FC. In addition, plants grown on 12.5% FC showed the lowest area with ‘low’ chlorophyll fluorescence, followed by plants grown under 25 and 100% FC. In addition, plants grown in 25 and 50% FC had a larger proportion ‘medium’ relative chlorophyll fluorescence area compared with controls, while plants grown at 25% FC showed the largest area with ‘high’ chlorophyll fluorescence, which is an indicator of leaf senescence (Fahlgren et al. 2015b).
**Water limitation effect on leaf relative water content**

The NIR camera of the HTPP platform and LemnaGrid software gave us the ability to quantify *in planta* water content and distribution. As shown in Fig. 8, all levels of leaf relative water content showed significant differences across treatments \( P < 0.0001 \). For the ‘low’ relative water content category, plants grown at 12.5 and 25% FC were statistically different than those grown at 50% FC and control. Plants grown at 100% FC showed the lowest value for ‘low’ relative water content when compared with all other treatments. Plants grown under severe water limitation (12.5 and 25% FC) were statistically different in ‘medium’ relative water content compared with those grown at 50 and 100% FC. Similarly, plants grown at 100% FC, showed the lowest value for ‘medium’ relative water content compared with the rest of the treatments. As an overall trend, the greatest amount of relative water content was obtained for plants grown under 100% FC whereas plants grown under severe water limitation showed the lowest values of leaf relative water content.

**Water limitation effect on photosynthetic efficiency**

Water limitation has been shown to negatively affect photosynthetic efficiency (PE) and chlorophyll fluorescence and linear electron flow (LEF) are parameters that can be used to assess the plant PE. For instance, the efficiency of PSII is directly related to the rate of LEF and inversely related to the chlorophyll fluorescence (Baker *et al.* 2007). As illustrated in Fig. 9a, plants grown under 100% FC had the highest linear electron flow values compared with plants grown at 50, 25 and 12.5% FC. However, we did not detect significant differences for any of the rest of photosynthetic parameters measured with the MultispeQ (\( \Phi II, NPQ, vH^+, gH^+, ECSt; \) data not shown).

**Seed yield is affected during water limitation**

It is well known that the stress caused by limited water availability induces yield reduction in many plant species, and this reduction depends on the severity and duration of the stress (Farooq *et al.* 2009). To assess the impact of water limitation on seed yield in *Arabidopsis*, we let the plants grown in all water saturation conditions to reach maturity and complete their life cycle. Seeds were then collected and counted on a per plant basis. We found that even under moderate water limitation conditions (50% FC) there is a significant reduction in the seed yield (Fig. 9b). Plants grown under the most severe water limitation treatment (12.5% FC) displayed the lowest seed yield of all.
Fig. 4. Representative images obtained using the Scanalyzer HTS (VIS, FLUO and NIR cameras) of *Arabidopsis* plants growing under four water regimes 100, 50, 25 and 12.5% water saturation for 29 days. (a, c, e) and (g) represent the original images obtained from the Scanalyzer HTS and (b, d, f, h) the analysed images after using the LemnaGrid software. Colour scale from FLUO images represented by black no, red low, blue medium and green high fluorescence. Colour scale from NIR corresponds to blue high, green medium and orange low water content.
Elemental analysis under water limitation regimes

Macro- and micronutrients are critical for several metabolic processes and also to maintain ion and osmotic balance during water limiting conditions. To assess the impact of water limitation on the leaf ionome, leaf samples from plants grown under the different water regimes were harvested at day 29 after germination to determine their elemental composition. At day 29, but not before, significant differences were found in leaf area (Fig. 5a) and leaf number (Fig. 6); therefore, we reasoned this could be a relevant point for elemental analyses. The concentration of Ca, K, Na, Mg, Mn, Fe, Zn and Cu in leaf samples was determined by ICP-OES as previously reported (Mendoza-Cózatl et al. 2014). The concentration of all elements was originally calculated as micrograms of element per milligram of DW and these data can be found in Fig. S1, available as Supplementary Material to this paper; however, to facilitate the analysis and include macro and micronutrients in the same graph, the element concentration was normalised to the values of plants grown at 100% FC (Fig. 10). Significant changes were found in all water regimes tested and more specifically three trends were identified. First, Ca, Mg, Na showed a similar pattern where a decrease in their concentration was found at 50% FC but, surprisingly, the concentration of these elements was similar to control plants or even higher when the stress became more severe (25 and 12.5% FC). This is particularly notable for Na (with a net increase to 27% respect to control plants grown at 12.5% FC) (Fig. 10a). Second, for the micronutrients Cu and Mn, we did not observe any decrease in their concentration in any condition tested. On the contrary, the concentration of both elements gradually increased with the reduction on water saturation reaching 30% (Cu) and 40% (Mn) higher concentrations, at 12.5% FC, compared with control plants (Fig. 10b). Third, we detected a significant decrease at 50 and 25% FC in the Fe concentration; however, at 12.5% FC Fe levels were not different that control plants. It should be noted that these data were originally calculated on a dry weight basis before being normalised and that plants grown at lower water saturation regimes had a severe reduction in biomass and this has to be considered when interpreting changes in the elemental composition throughout the different water regimes.
Finally, the concentration of K and Zn remained constant in all conditions tested (Fig. S1).

**Discussion**

Quantification of plant phenotypes by HTPP approaches is essential to take full advantage of natural variation, -omic technologies and molecular breeding approaches with the ultimate goal of crop improvement. The images acquired with HTPP platforms are rich in information in the sense that they contain and describe all the major characteristics of plants and the dynamics of their response to changes in environmental conditions, including biotic and abiotic stresses (Junker *et al.* 2015). In this study we describe the methodology to obtain HTPP images on an optimised water limitation assay using the reference plant *A. thaliana*. High-throughput phenotyping allows entire experiments to be accurately quantified in a non-destructive manner, meaning that changes in plant growth throughout the life cycle can be documented temporally. Moreover, by identifying critical shifts on plant growth and development patterns, additional high-throughput technologies

![Fig. 6. Water availability reduces the leaf number in Arabidopsis. Changes in leaf number under four different water regimes 12.5, 25, 50 and 100%. Data are means ± s.e. (n = 15). Different letters represent significant differences between treatments (*P* < 0.0001 at CI 95%).](image1)

![Fig. 7. Impact of water limitation on chlorophyll fluorescence. Chlorophyll fluorescence expressed as relative to projected leaf area as an indicator of tissue health in plants grown under 12.5% (a), 25% (b), 50% (c) and 100% (d) soil saturation conditions. Data represent the means of 30 replicates. Significant differences were found between 12.5, 25, 50 and 100% water saturation (*P* < 0.0001 at CI 95%).](image2)
such as elemental profiling (or ionomics), fluxomics, transcriptomics or proteomics can be implemented to gain further insight into the molecular mechanisms that led to the original phenotype. These analyses may also shed light on processes that may have a delayed impact at later stages of developmental, including yield or nutritional composition of grains. Here, we explored the combination of two high-throughput methods, phenomics and ionomics, to begin documenting the effects of water limitation at a resolution that would have been difficult to obtain using stand-alone or manual independent measurements (Figs 1, 2).

Soil water potential is a good indicator to understand from the plant physiology perspective how difficult it would be for a plant to take up water from their surroundings (Jones 2007). Our measurements show that our water limitation regimes, expressed as a percentage of water holding capacity, correspond to water potential values that have been previously considered as moderate to severe water deficit regimes (−0.5 MPa to −1.6 MPa; Fig. 3) (Bhaskara et al. 2015; Durand et al. 2016). Having standardised water deficit regimes is important to obtain comparable results even if experiments are performed in different laboratories and deviation from these values may impact the ionomic and phenomic data acquired through the experiments. At the physiological level, it is accepted that water deficiency may lead to growth arrest, cavitation in the xylem vessels (Zufferey et al. 2011), stomatal closure, cessation of water and nutrient transport and carbon re-allocation and eventual starvation, all of which threaten plant survival (McDowell et al. 2008). In agreement with these observations, our data shows that reduced water availability has a negative impact on plant growth and development (Figs 4, 5). Notably, the negative effects were treatment-dependent and time-dependent. For instance, differences in the projected leaf area were only significant 10 days after the water limitation regime started and these differences became more obvious between treatments as the experiment progressed over time (Fig. 5a).

An additional indicator of the plant metabolic status is chlorophyll fluorescence. In optimal conditions, plants exhibit a basal level of chlorophyll fluorescence from absorbed light and the majority of this energy gets directed towards photosynthesis. When plants experience abiotic stresses, such as water limitation, the balance between photosynthetic efficiency and energy dissipation as heat and fluorescence increases, resulting in particularly high levels of fluorescence (Muller et al. 2001). Our results show that medium and high relative chlorophyll fluorescence are largest in plants grown in the two lowest saturation regimes (25–12.5% FC), indicating

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**Fig. 8.** Relationship between soil water saturation and plant water content. Water content results expressed as relative to projected leaf area as an indicator of tissue health in health in (a) 12.5%, (b) 25%, (c) 50% and (d) 100% water saturation treated wild type *Arabidopsis italiana* plants. Data represent the means of 30 replicates. Significant differences were found between 12.5, 25, 50 and 100% water saturation ($P < 0.0001$ at CI 95%).
that water limitation negatively affected the photosynthetic efficiency of the plants (Fig. 7).

Photosynthetic activity can also be determined by measuring the flow of available electrons used to produce ATP and NADPH (Foyer et al. 2012). When water is limited, the stress causes a reduction in carbon fixation due to stomatal closure, which will cause an over-reduction of the electron transport chain intermediaries (Golding and Johnson 2003). In addition, biomass accumulation can also be stunted when plants are subjected to water limitation. Schuppler et al. (1998) showed that leaf area and the number of leaves is reduced in response to water deficiency, which in turn will reduce water availability and ultimately leading to biomass and yield loss. Accordingly, our data show that plants grown under control conditions (100% FC) produced more leaves than the plants growing under water deficit (Fig. 6). In addition, control plants had a higher linear electron flow compared with those growing under water limitation stress, indicating that water availability allowed plants to thrive and support a larger number of leaves while maintaining a high photosynthetic efficiency (Fig. 9a). Water limitation has been shown to negatively impact crop yield.

In agreement, our data show that even moderate water limitation conditions (50% FC), led to a significant reduction on seed yield (Fig. 9b), decreasing by more than 3-fold the amount of seeds produced per plant.

As predicted from previous studies, water limitation in the soil led to lower water content in leaves (Bartels and Sunkar 2005). By visual inspection, water deficit symptoms such as chlorosis or necrosis may have been missed throughout the water limitation experiment (Fig. 3b). However, the NIR camera (Fig. 8) was able to detect changes in water content in planta, and plants grown under normal conditions showed a greater amount of available water. In addition, Fig. 4 shows the pattern in which water is distributed in the plants, including the fact that leaves had lower water content at the edges. This occurs because these areas have the least amount of moisture in the leaf.

As for the elemental composition of plant tissues during water limiting stress, it has been generally assumed that water limitation restricts the bulk flow movement of nutrients through
the soil matrix into the roots, thus reducing the overall uptake and accumulation of minerals in plant tissues (Kramer and Boyer 1995). This effect may be counteracted by the fact that water limitation also restrict dry mass accumulation (i.e. shoot growth) and this could in turn compensate for the reduced uptake of minerals into the plant (Kramer and Boyer 1995). Our elemental profiling results however, show that the water limitation treatment affects the elemental composition of leaves in an elemental- and treatment-specific manner (Fig. 10). At the macronutrient level, Ca, Mg and Na seem to decrease, on a DW basis, at the 50% FC (Fig. 10a). However, the values were similar, or in the case of Na, even higher compared with control plants at lower water availability regimes. In Arabidopsis, Na is considered as a non-essential element but its over-accumulation has been associated with a higher capacity to sustain water retention, thus improving the ability of plants to cope when water availability is scarce (Gaxiola et al. 2001). Some micronutrients, on the other hand, show particularly defined patterns across water limitation regimes. For instance, Mn and Cu remained at level similar to control plant values at moderate FC levels and even increased at severe water limitation regimes (Fig. 10b). Fe levels on the other hand, were decreased at 50 and 25% FC but unexpectedly, the Fe levels in plants grown under 12.5% FC were no different from those of control plants. Fe is critical for photosynthesis but at high concentration it becomes toxic due to the generation of reactive oxygen species (ROS) through the Fenton reaction (Matros et al. 2015). During water limitation photosynthesis is impaired and the accumulation of reducing intermediaries could become even more detrimental in the presence of Fe, leading to a higher rate of ROS production. Therefore, keeping Fe at low levels during water limitation may be a plant response to prevent further oxidative stress. Mn and Cu, on the other hand, are critical for the water-splitting and electron transfer reactions in photosynthesis (Suorsa and Aro 2007) and since the photosynthetic efficiency of plants is impaired during water limitation, upregulation of Mn and Cu uptake and accumulation systems may be an attempt to maintain or improve photosynthetic efficiency. Over-accumulation of Mn during drought has been previously observed in several Arabidopsis accessions (Ghandilyan et al. 2009). Moreover, Mn is a critical transition metal for ROS detoxification by Mn superoxide dismutases (Mn SOD); therefore, the additional Mn in plants experiencing water limitation may be funnelled towards Mn SOD for a more efficient ROS detoxification. In agreement with this hypothesis, Mn SOD has been found to be upregulated in drought conditions (Alscher et al. 2002). As a whole, these results also reinforce the notion that plant responses are strictly stress-dependent and, more specifically, that plant responses depend on the severity of the stress imposed. Therefore, we should be cautious about generalising the effects of water limitation on the elemental composition of plants and other phenotypes.

Conclusions
Our HTPP platform allowed us to assess a water limitation protocol that now can be used as an experimental pipeline to study plant responses to water limitation in a controlled and reproducible environmental setting. Reproducibility is critical for future experiments that may include additional high throughput technologies at specific time points and gain further insight into molecular and physiological responses of plants undergoing biotic and abiotic stresses. Our data also show that plants grown under mild stress (50% FC) grew at the same rate than control plants; however, a clear penalty was observed in photosynthetic efficiency and seed yield. In addition, water limitation stress led to a reduced shoot growth and overall biomass, determined as a reduced leaf number. When water limitation was more severe, all the physiological and metabolic parameters measured were negatively impacted even further. We also demonstrated the utility of the HTPP platforms to generate quick, accurate and large non-biased datasets that can be used to determine the overall plant fitness and performance. Accurate quantification of subtle and novel physiological responses to abiotic stresses allow researchers to document time-dependent changes that otherwise would be undetectable to the naked eye in a non-destructive and high-throughput manner. In addition, we also demonstrate the utility of HTPP platforms to identify critical points, were additional high throughput technologies such as elemental profiling, can be implemented to get a better understanding of the plant physiological responses to abiotic stresses such as water limitation. Linking high throughput technologies in a systematic fashion will facilitate data integration and interpretation, thus offering a better opportunity to unravel the still largely unknown mechanisms that plants use to thrive on challenging environments. Understanding these mechanisms is critical to developing crops capable of sustaining growth and yield in times when global climate change poses a serious threat to food security worldwide.

Acknowledgements
This work was supported by the Plant Imaging Consortium (http://plantimaging.cast.uark.edu/) funded by the National Science Foundation Award Numbers IIA-1430427 and IIA-1430428. The HTPP platform was acquired with funds from the Arkansas Centre for Plant Powered Production (http://www.plantpoweredproduction.com/) through the RII Arkansas ASSET Initiative (Arkansas EPSCoR) by NSF grant # EPS-0701890.

References