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Investigating root architectural differences in lines of *Arabidopsis thaliana. L*. with altered stomatal density using high resolution X-Ray synchrotron imaging

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Abstract

Purpose Freshwater is an increasingly scarce natural resource, essential for agricultural production. As plants consume 70% of the world's freshwater, a reduction in their water use would greatly reduce global water scarcity. Plants with improved Water Use Efficiency (WUE) such as those with altered expression of the Epidermal Patterning Factor (EPF) family of genes regulating stomatal density, could help reduce plant water footprint. Little however, is known about how this modification in *Arabidopsis thaliana. L*. affects root architectural development in soil, thus we aim to improve our understanding of root growth when stomatal density is altered.

Methods We used X-Ray synchrotron and neutron imaging to measure in three dimensions, the root system architecture (RSA) of *Arabidopsis thaliana.*

L. plants of three different genotypes, namely that of the wild type Columbia (Col 0) and two different EPF mutants, EPF2OE and *epf2-1* (which show reduced and increased stomatal density, respectively). We also used the total biomass and carbon isotope discrimination (Δ) methods to determine how WUE varies in these genotypes when grown in a sandy loam soil under controlled conditions.

Results Our results confirm that the EPF2OE line had superior WUE as compared to the wild type using both the Δ and total biomass method. The *epf2-1* mutant, on the other hand, had significantly reduced WUE using the Δ but not with the biomass method. In terms of root growth, the RSAs of the different genotypes had no significant difference between each other. There was also no significant difference in rhizosphere porosity around their roots as compared to bulk soil for all genotypes.

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Conclusion Our results indicate that the EPF mutation altering stomatal density in *Arabidopsis thaliana. L.* plants did not have an adverse effect on root characteristics thus their wide adoption to reduce the global freshwater footprint is unlikely to compromise their soil foraging ability.

Keywords *Arabidopsis thaliana. L.* · X-Ray synchrotron imaging · Root System Architecture · Water use efficiency · Neutron imaging

Abbreviations

WUE	Water Use Efficiency
EPF	Epidermal Patterning Factor
SXCT	X-Ray Synchrotron Computed Tomography
NCT	Neutrons Computed Tomography
Carbon isotope discrimination	Δ
RSA	Root system Architecture

Introduction

Persistent water shortages and scarcity are major global challenges that affect the livelihoods of more than half of the world's population, many of whom live in the least developed parts of the world (FAO 2020; Miletto 2021). Prudent use of the increasingly limited freshwater resources available especially in water scarce regions is necessary to maintain food security and general quality of life (Zahoor et al. 2019; Cao et al. 2021; Gleick and Cooley 2021). As agriculture is predominantly the largest withdrawer of global freshwater resources, accounting for close to 70% of all freshwater withdrawals, strategies that maximise agricultural water utility are paramount to reducing global water scarcity (Hoogeveen et al. 2015; Gleick and Cooley 2021). Reducing water used for irrigation of crop plants is one such promising strategy that could help optimise agricultural water use. This is because irrigation accounts for the bulk of the water used by modern agriculture and as such, even a marginal decrease in plant water use could result in a noticeable decline in agricultural freshwater consumption (de Avila et al. 2015; Cao et al. 2021).

Numerous strategies to improve the efficiency of freshwater use by plants such as breeding to increase harvest index, deficit irrigation and partial root zone drying have been developed with some showing great promise for enabling a more sustainable path for agricultural water use management (Morison et al. 2008; Ullah et al. 2019). Of these interventions, one of the most interesting is through the genetic manipulation of stomatal density, which has been shown to improve both drought resistance and Water Use Efficiency (WUE) in plants through limiting transpiration, which is the main source of water loss from plants (Bertolino et al. 2019; Buckley et al. 2020). Reducing stomatal density by manipulating the expression of different members of the Epidermal Patterning Factor (EPF) family of genes has been demonstrated to confer improved drought resistance and water use efficiency. This has been shown to be the case in *Arabidopsis thaliana. L.* (Franks et al. 2015; Hepworth et al. 2015, 2016), barley (Hughes et al. 2017), rice (Caine et al. 2019; Mohammed et al. 2019) and wheat (Dunn et al. 2019). The wide use of this strategy could potentially decrease the water footprint associated with plant productivity and thus have a great impact in dealing with the prevalent global water shortages.

Despite the beneficial traits of manipulating the EPF genes, little is known about how changes in stomatal density affect root architectural development. This is in-spite of the fact that the alterations in stomatal density result in changes in plant transpiration which has a direct bearing on soil resource acquisition (the chief responsibility of roots) and as a consequence this may affect root behaviour. Some studies like Mohammed et al. (2019) have shown how changes in the EPF1 gene homologue in rice OsEPF1 increases rice root aerenchyma. Hepworth et al. (2016) also showed evidence of a reduction in total root length as a result of the overexpression of the EPF2 gene in *Arabidopsis thaliana. L.* The deficit in studies looking at root properties is mainly due to the fact that roots are often obscured within opaque soil and thus are frequently neglected as they are not easily studied using traditional methods of plant analysis. This is even more apparent in the model plant, Arabidopsis, which is a relatively small plant whose root properties when grown in soil are seldom measured in literature. In many of the Arabidopsis experiments, roots are often grown in colourless growth media

such as agar gel (French et al. 2009; Xiao et al. 2015), hydroponic solutions (Dayod et al. 2013; Strehmel et al. 2014) or even artificially synthesised transparent "soil" (Downie et al. 2012; Ma et al. 2019). Although these techniques provide useful insights into how specific gene alterations may affect root growth, they lack a specific dimension patterning to the actual performance of *Arabidopsis thaliana. L.* roots in soil, their natural growth media.

Studying *Arabidopsis thaliana. L.* root properties in soil using the traditional method of root washing is difficult due to the fact that *Arabidopsis thaliana. L.* roots are very thin (48–150 μm thickness) and fragile, resulting in significant losses during the washing process. As an alternative, some researchers have resorted to using angled plastic rhizotrons with uniformly graded sand, encouraging the roots to grow at the glass sand interface for ease of visualisation (Chapman et al. 2011; Hepworth et al. 2016) used vermiculite containing rhizotrons covered with a glass microfiber paper that prevented direct contact between *Arabidopsis thaliana. L.* roots and the growth media thus enabling easy separation and analysis of the roots. All these techniques however do not give a true representation of how *Arabidopsis thaliana. L.* plants would grow in natural environments. To counter for the challenges faced by the traditional root-soil analysis methods, Lucas et al. (2011), Seignez et al. (2010), Tracy et al. (2010) and more recently Morris et al. (2017) used more advanced X-Ray CT scanning to reveal *Arabidopsis thaliana. L.* roots in natural soil. Most of these studies, however, did not reveal the entirety of the root architecture of the *Arabidopsis thaliana. L.* plant. In the case of Lucas et al. (2011) (scan resolution 18 μm pixel^{-1}), the researchers resorted to using other non-soil based methods to quantify root architectural properties in the mutants they studied whilst Tracy et al. (2010) (scan resolution 16 μm pixel^{-1}) only as a proof of concept showed only a single grayscale image of an *Arabidopsis thaliana. L.* without attempting to segment out the roots in the image. Seignez et al. (2010) (scan resolution 10 μm pixel^{-1}) on the other hand in a soil pollution study investigated only a small root section of *Arabidopsis halleri* (Similar genus to *Arabidopsis thaliana*) in contact with contaminated soil as opposed to revealing the entire root architecture of the plants grown. Only Morris et al. (2017) (scan resolution not specified) in a review looking at how roots

are shaped by different stimuli by goes into greater detail of *Arabidopsis thaliana. L.* root architecture. In this study they looked at a time series of growth of an Arabidopsis seedling growing in soil for a 21 day period. Even in this experiment however, less than half of the soil core was scanned to reveal root architecture, leaving out a significant portion of the growing roots unimaged.

In-spite of the challenges associated with studying *Arabidopsis thaliana. L.* in mineral soil as outlined above, here we aim to use soil based systems to better understand the consequences of alterations in stomatal density via manipulation of the EPF family of genes on root properties in *Arabidopsis thaliana. L.* This study compares three different *Arabidopsis thaliana. L.* lines with differing stomatal densities; the wild-type control (Col-0), *epf2-1* (a mutant that has an increased stomatal density) and EPF2OE (a transgenic line that overexpresses *EPF2* gene resulting in greatly reduced stomatal density). Our specific objectives were to (a) Estimate the WUE of the EPF mutants as compared to wild type plants when grown under controlled conditions in soil using biomass and Δ methods, (b) Reveal whole root architectural properties of the different *Arabidopsis thaliana. L.* lines when grown in soil using high-resolution synchrotron imaging scanning as well as Neutron CT.

Methods and materials

Plant growth conditions

Three *Arabidopsis thaliana* (L.) Heynh genotypes were used in this study. These were the wild type Col-0 (used as control) and two stomatal mutants *epf2-1* and EPF2OE all derived from the Col-0 background as described in Hunt and Gray (2009). Prior to plant growth, seeds were stratified at 4°C for 48 h before being transferred to a controlled growth chamber maintained at a temperature of 22 °C (day)/18°C (night) (standard deviation ± 2 °C) and at a relative humidity of 55% with a 9 h day length. A sandy loam soil (70% Sand, 16% Clay and 14% Silt) obtained from Cove farm (53°30'03.7"N 0°53'57.2"W) with an organic carbon content of 4.18 ± 0.18% was used in this study. This was sieved over a 2 mm sieve to eliminate coarse particles and packed in the necessary pots/containers to a bulk density of 1.2 g.cm^{-3}.

The plants were grown at a distance of 30 cm (maximum) from the light source receiving light averaging 180 μmol m^{-2} s^{-1} (±15 μmol m^{-2} s^{-1}) in intensity at plant height. Watering was done every 3–4 days to ensure that the water content was maintained a volumetric water content between 20 and 25% for the duration of the growth experiments. The *Arabidopsis thaliana. L.* plants used were grown for between 6 and 8 weeks depending on the analysis to be done after their growth. The number of replicates for the different plant genotypes grown were dependent of the experiment conducted with more than five plants being grown for each experiment and at least three of these being used for the different measurements.

Stomatal density

Stomatal impressions using resin (Coltene, PRESIDENT) were done on the abaxial surface of three fully expanded leaves per plant with at least four plants of each genotype used. These impressions were embedded on a microscope slide using clear tape and Z-stack images were taken under a Leica DM IRBE Inverted Microscope (20x/ 0.4∞/ 0.17-A). Z-stack files obtained from microscopy were opened using ImageJ software (v.1.52p) and numeration of abaxial stomata was done using the count tool. As stomatal properties vary along the leaf surface, to attain a more uniform characterisation of stomatal properties, the leaves were divided into three equal sections as illustrated in Fig. 1. The total number of stomata on a section covering an area 400×400 pixels (185 μm^2) in the field of view of the microscope was counted for each of the different sections. The stomatal density (SD) for each leaf was calculated as the average number of counted stomata across the three different sections per mm^2.

Water use efficiency estimation

WUE in this study was estimated using two different methods, namely using the ratio between total dry biomass and water lost via evapotranspiration (determined gravimetrically) as well as the Δ method (Farquhar et al. 1989; Bacon 2009). In the biomass ratio method, the total dry biomass was measured after drying all the plant tissues at 60°C for 48 h and the total amount of water lost via evapotranspiration during the growth of a

Fig. 1 Sections of the *Arabidopsis thaliana. L.* leaves where the different cell counts were made
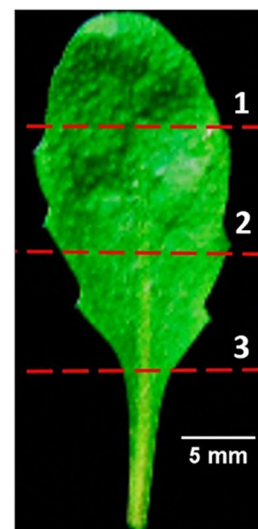
plant. To reduce soil surface evaporation, a transparent plastic sheet was placed on the surface of the soil surrounding each plant, this allowed us to measure plant WUE more accurately for each plant. The Δ method, on the other hand involved using plant leaves to determine the WUE of a particular plant, these were sampled from at least 3 mature leaves from each plant at the same developmental stage for each different plant genotypes. The sampled material was oven-dried at 65 °C for 48 h then ground using a ball bearing in a tissue lyser machine to a fine powder. This powder was then used to determine the ^{13}C/^{12}C carbon ratio using an ANCA GSL 20–20 Mass Spectrometer made by Sercon PDZ Europa (Cheshire, United Kingdom). The sample δ^{13}C results reported by the machine were not absolute measurements but indicated the difference between our given samples (R_{sample}) vis a vis the standard Vienna Pee Dee Belemnite (PDB) ($R_{standard}$). The sample δ13C was then calculated using the formula specified in Farquhar et al. (1989) that is as follows:

$$\delta^{13}C_{sample} = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000 \qquad (1)$$

The C isotope discrimination (Δ) of each plant sample was then calculated using the formula:

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{sample}}{1 + \delta^{13}C_{sample}} \qquad (2)$$

Where $\delta^{13}C_{air}$ was obtained from at least five samples of air taken from the growth chamber where the plant material analysed was grown.

Synchrotron X-Ray computed tomography (sX-ray CT)

The *Arabidopsis thaliana. L.* plants used in the sX-ray CT experiments were grown for 26 days after sowing in cylindrical polyvinyl chloride (PVC) tubes (36 mm diameter, 70 mm height). sX-ray CT was done using the I12-JEEP beamline (Drakopoulos et al. 2015) at the Diamond Light Source facility (UK) using a monochromatic beam with an image resolution of 7.91 μm per pixel (FOV = 20 mm×12 mm). A double field of view scan was employed with a total of eight scans being done to cover the entire core used for plant growth as illustrated in Fig. 2. A total of 11 cores were scanned with at least three scans being done for each genotype. Tomographic reconstruction was performed using the SAVU system (Atwood et al. 2015; Wadeson and Basham 2016). A filtered back projection (FBP) algorithm was also used (Ramachandran and Lakshminarayanan 1971), as

implemented in the ASTRA toolbox (van Aarle et al. 2016).

Neutron tomography

As an alternative method for imaging *Arabidopsis thaliana. L.* root architecture that would make root segmentation easier, we used neutron tomography. This imaging method is often considered complementary to X-Ray tomography as it can accentuate subtle low density components such as water (contained in roots), that are not easy to identify in X-Ray CT scans. The tubes used for X-ray CT were unsuitable for measurements with neutron imaging as they were made of PVC plastic which is highly neutron attenuating. As a result we used aluminium cylinders (10 mm diameter, 140 mm height) for neutron computed tomography (NCT) measurements. The *Arabidopsis thaliana. L.* plants grown were grown for 28 days. Neutron imaging was carried out at the IMAT neutron imaging and diffraction beamline of the ISIS neutron spallation source at the Rutherford Appleton Laboratory (UK) (Burca et al. 2018) using an optical camera box equipped with a 2048×2048 pixels (each pixel of 55 μm) Andor Zyla 4.2 PLUS sCMOS camera for a standard white beam neutron tomography

Fig. 2 Image showing the 8 different regions covered by each scan which were subsequently reconstructed into a single stack
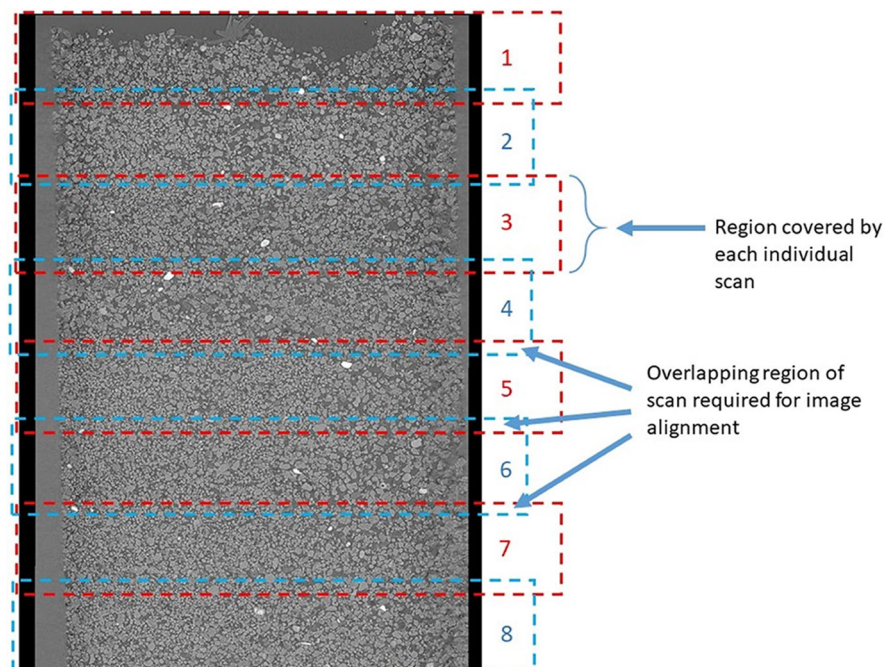
with 0.7-7 Å wavelength range. Scanning was performed following a modified version of the protocol similar to that described in Mawodza et al. (2020). The scanning of the cylindrical containers was done with a rotation step of 0.913° for each projection with an exposure time of 30 s per radiography resulting in 394 projections over 3 h of measurement. Two samples were scanned simultaneously with a multiaxial tomography stage equipment available at ISIS (Burca, G., Private communication, 8 February 2020).

Image processing and analysis

The reconstructed scans from both sX-ray CT and NCT were saved as two dimensional cross sectional images of the scanned plants which were imported into either Image J (Schneider et al. 2012) or Avizo 9.0.1 (FEI 2015) for further processing. The eight individual stacks for each plant from sX-ray CT were aligned and merged into a single stack. As noise was minimal and image sizes were large (> 20 Gb) in the sX-Ray, a 3×3 median filter which required minimal processing was used prior to root segmentation. On the other hand, for NCT images, a non-local means filter (with a search window of 21 pixels, local neighbourhood of 5 pixels and similarity value of 0.6) was used to eliminate noise prior to segmentation. Automated root segmentation techniques using algorithms such as Root1 in ImageJ as well as Rootrak were attempted but these were unable to successfully segment out the roots from sX-ray CT (Mairhofer et al. 2012; Flavel et al. 2017). As a result we used a combination of manual tracing which involves physically selecting root pixels from each of the slices that make up the 3D image as well as the interpolating algorithm in Avizo to extract the entire root architecture. As roots were clearer in NCT, the majority of the segmentation was carried out using the Root1 algorithm in ImageJ. The results of this segmentation were imported into Avizo and additional manual segmentation was carried out to enhance the automated segmentation. Due to the considerable moisture within the tubes used for NCT however, extracting the entire root system was not possible. For sX-ray CT root length, lateral root number, root thickness, convex hull and root volume were determined using the modules in Avizo whilst root angle was determined

in an exported BMP file using the Rooth software. Root angle in this study was defined as the angle between the horizontal plane and the long axis of each root, whilst convex hull was defined as the smallest convex set of pixels that contains all other pixels in the root system (Oyanagi 1994; Iyer-Pascuzzi et al. 2010). This volume can be used to define the potential for soil exploration of the soil by a root system (Helliwell et al. 2019). The evolution of root length density with depth for each of the different plants was determined by dividing the plant pots into three equal sections, each 2 cm in depth and subsequently determining the root length in each section. This was then divided by the soil volume to determine root length density.

Statistical analysis

The analysis of the results from these experiments was carried out using Graphpad prism v9.0 (Graph-Pad Software, LLC). Assumptions of normality and homogeneity of variance were performed using Shapiro-Wilk and Bartlett tests respectively. A one way ANOVA was used to explore the difference in means with a post hoc Bonferonni test used to separate between means.

Results

Stomatal characteristics

Abaxial stomatal properties showed considerable differences between the genotypes as expected. SD (Fig. 3D) in the *epf2-1* mutant was around 300 stomata/mm^2 which was approximately 44% higher as compared to the wild type which had a stomatal density of 200 stomata/mm^2. On the other hand the EPF2OE mutant had a SD of 55 stomata/mm^2 which represented a reduction of 73% reduction in stomata as compared to the wild type.

Water use efficiency

In terms of water use efficiency, significant differences in Δ (Fig. 4A) were found between both the mutants as compared to the wild type plants with the epf2 mutant showing significantly increased Δ, indicating lower WUE whilst the EPF2OE lines showed
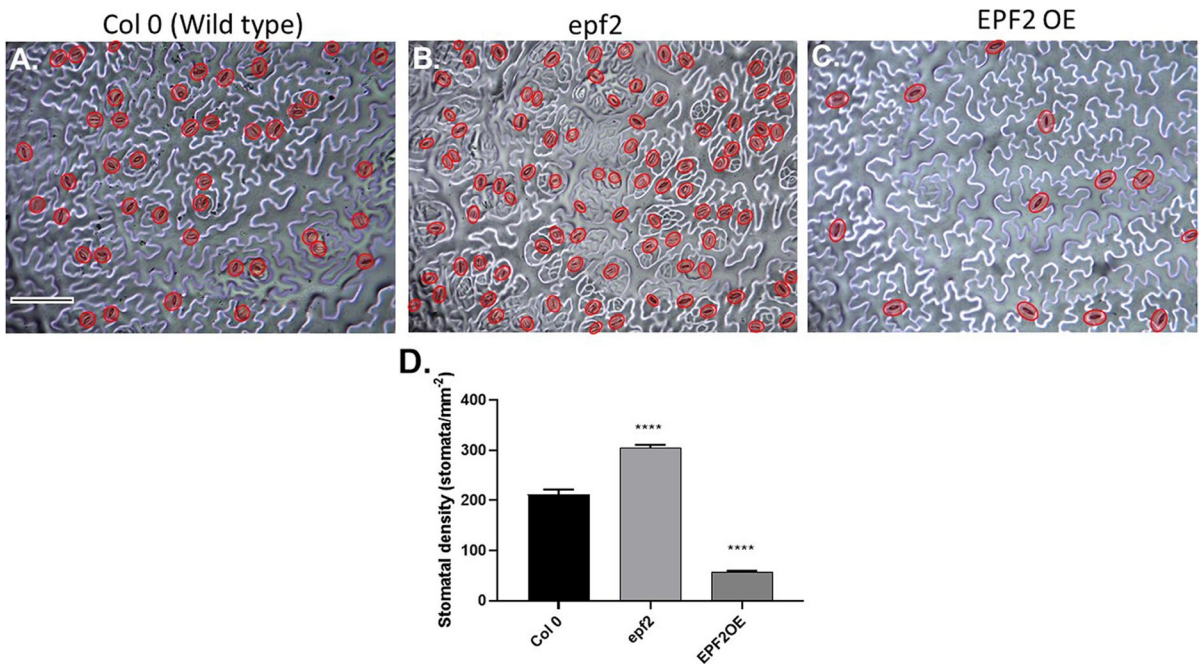
Fig. 3 **A**-**C** Stomatal density of the abaxial *Arabidopsis thaliana. L.* surface (bar indicates 100 μm). **D** Stomatal density *Arabidopsis thaliana. L.* surface of different mutants. Error bars indicate standard error of the Mean (SEM). Symbols indicate significant difference as compared to Col-0; (*=≤0.05, ****= ≤0.0001)

significantly reduced Δ indicating improved WUE. Complimentary to this, WUE measurements in terms of biomass production per unit water loss (Fig. 4B) also showed a significant increase in WUE for the EPF2OE line plants over a 50 day growth period in comparison to the wild type plants. Unexpectedly, however, there was no significant decrease in WUE of the epf2 mutant as estimated using this method despite the significant increase in Δ. The WUE of this mutant was only marginally lower than that of the wild type plants. There was also no significant difference in the shoot dry biomass of the different genotypes (Fig. 4C) with EPF2OE having the lowest shoot biomass as compared to the other genotypes.
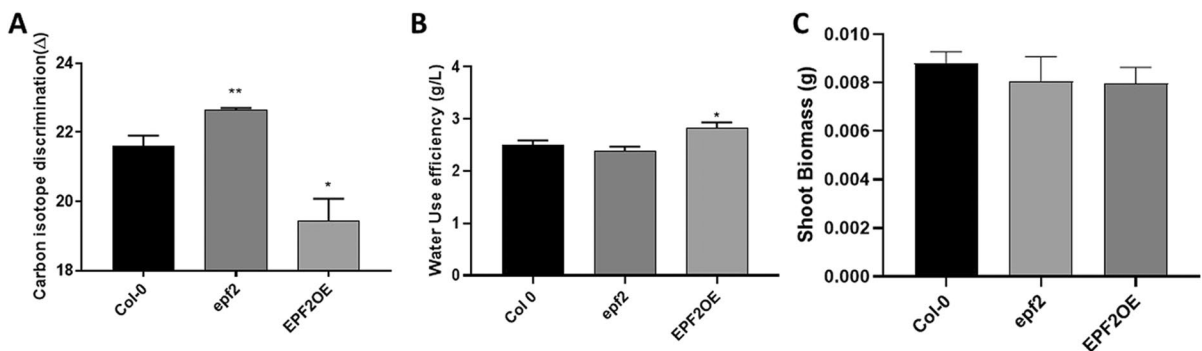
Fig. 4 Water use efficiency as estimated by (**A**) Carbon isotope discrimination and (**B**) Biomass to water transpiration in *Arabidopsis thaliana. L.* plants grown until maturity (n ≥ 3) and (**C**) Shoot biomass of the different genotypes at the time of imaging. Error bars indicate standard error of the Mean (SEM). Symbols indicate significant difference as compared to Col-0; (*=≤0.05. **= ≤0.01)

Root architectural properties

Determining root architecture from neutron imaging proved difficult as the contrast between *Arabidopsis thaliana. L.* roots and soil was not pronounced. Only parts of the primary root (Fig. 5) could be visualised in our experiment, which limited our inferences from NCT. On the other hand, images from sX-Ray CT, which had higher resolution, were able to reveal both primary and lateral roots in detail (Figs. 6). Our segmentation of sX-Ray CT images resulted in a detailed three dimensional root networks from which root properties could be determined with up to 2nd order lateral roots being segmented from our scans. There were no significant differences in root length, volume, diameter and angle between the different mutants as compared to the wild type (Fig. 7A-D). There was however a general trend however that the EPF2OE plants showed reduced root metrics for these properties with the epf2 mutant being in between the wild type and the EPF2OE. The most striking difference, however, was between lateral root densities of the EPF2OE mutants as compared to the wild type and *epf2-1* mutants. This was about 30–35% lower

Fig. 6 Three dimensional renderings of the different *Arabidopsis thaliana. L.* genotypes from sX-Ray CT scanning (Bar indicates 10 mm). Col-0 (wild type) roots are A,D and G, epf2-1 roots are B, E and H and EPF2OE roots are C, F, I

than that of the other genotypes. In terms of root length density (RLD) with depth (Fig. 8), this was quite similar for all the genotypes in the top 2 cm of the soil with an average of around 2 cm/cm^{-3} for all the plants and the wild type having the lowest surface layer RLD. In the middle Sects. (2-4 cm depth) however, this was reversed with the wild type plants having the highest RLD that was significantly higher than that of the *epf2-1* mutants. In the lowest Sects. (4-6 cm depth), there was marked decline in the EPF2OE mutant's RLD which was lowest (although not significantly lower) whilst the wild type and the *epf2-1* mutant plants had a relatively similar RLD.

Discussion

In this study, we examined the impact of altering stomatal density (and consequently WUE) on the root architecture of *Arabidopsis thaliana. L.* plants. We
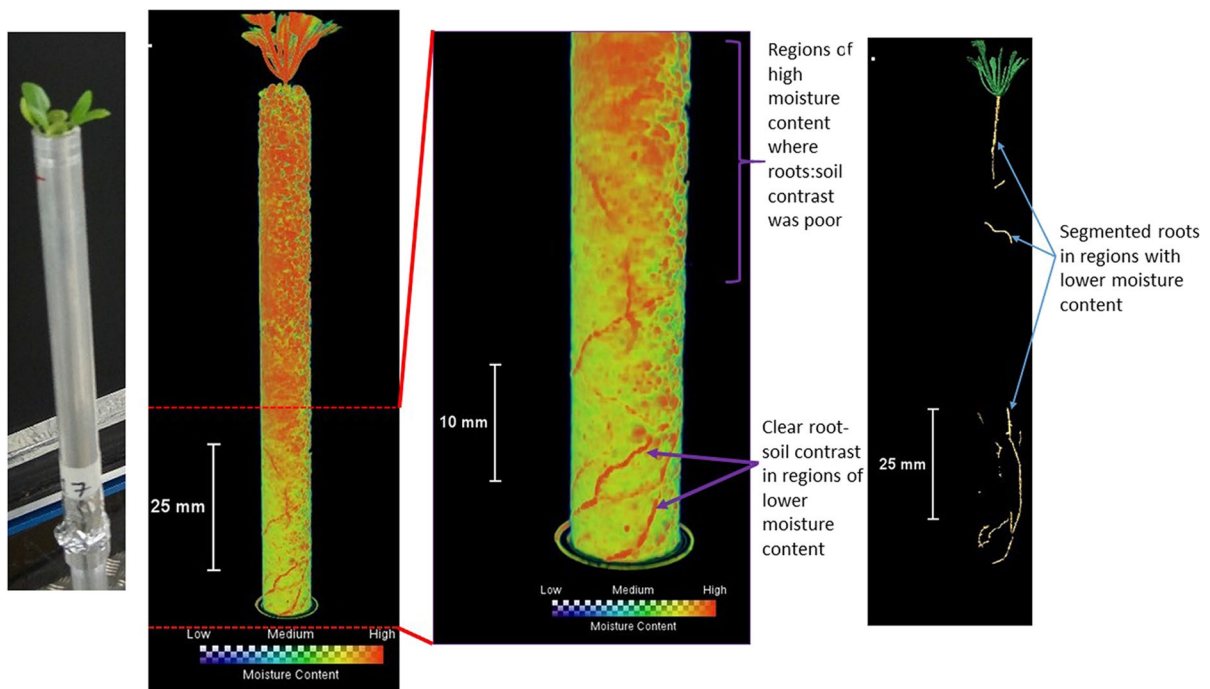
Fig. 5 *Arabidopsis thaliana. L.* root NCT image showing regions of high soil moisture (orange zones) which prevented accurate root segmentation. Roots distinct and large enough to segment are shown on the extreme right
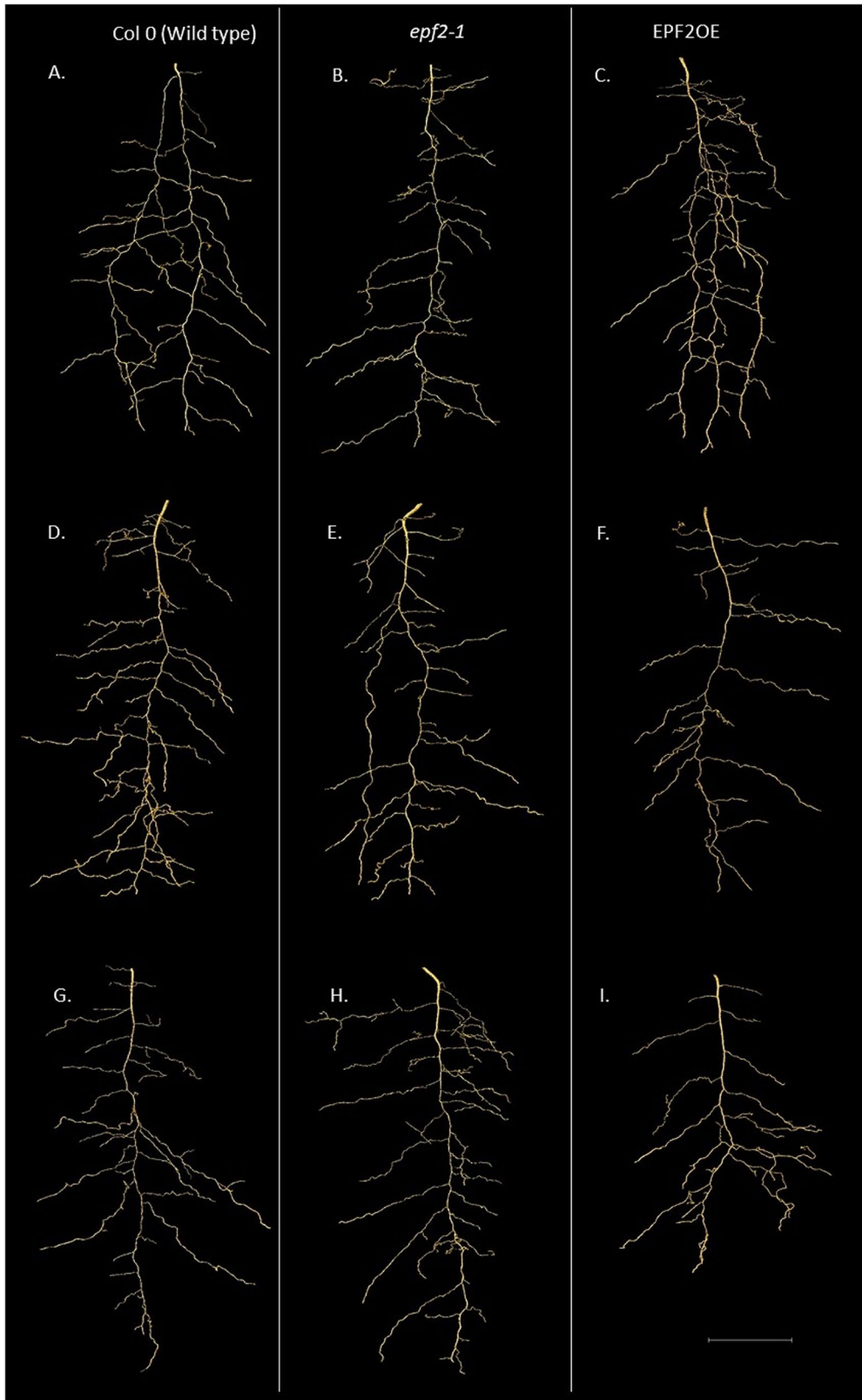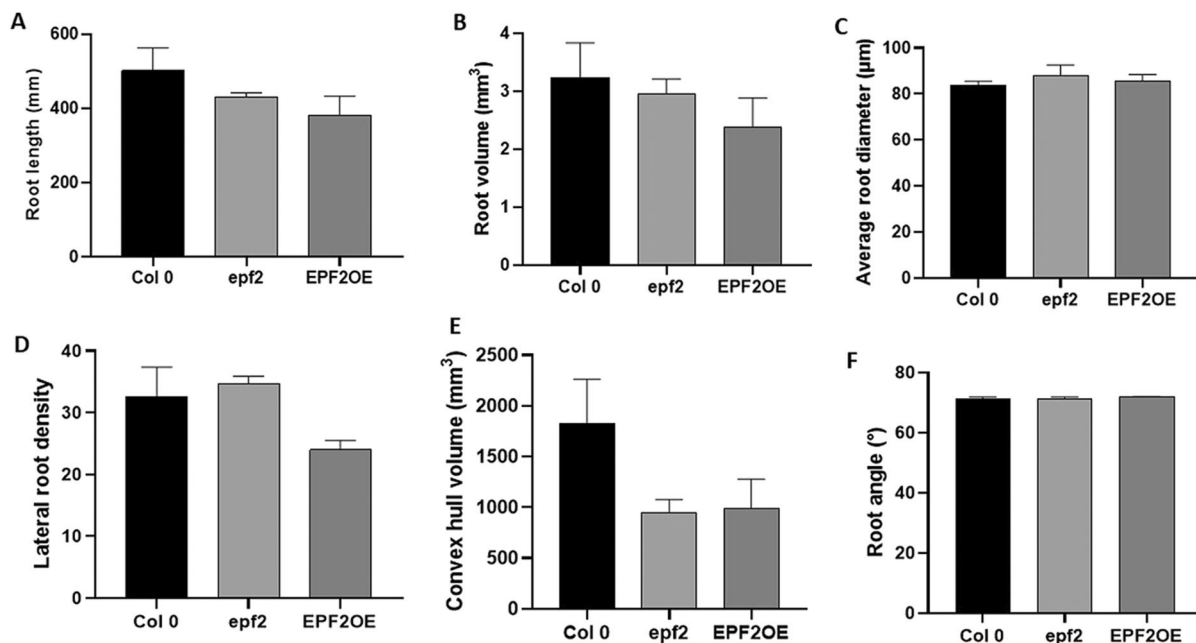
Fig. 7 Root properties of *Arabidopsis thaliana. L.* plants grown in a sandy loam soil as derived from sX-Ray CT, **A** Root length, **B** volume, **C** diameter, **D** Lateral roots per primary root, **E** convex hull volume, **F** lateral root angle (n = 3). Error bars indicate standard error of the mean (SEM)

were able to visualize Arabidopsis thaliana. L. RSA in much greater detail as compared to most other studies done previously as we took advantage of high energy synchrotron X-Rays to obtain high resolution (7.9 μm pixel^{-1}) scans of roots growing in a mineral soil. We were also able to make inferences on how the roots of EPF mutants interacted with soil structure as we obtained three dimensional maps of root-soil interactions for all the plants used in our study.
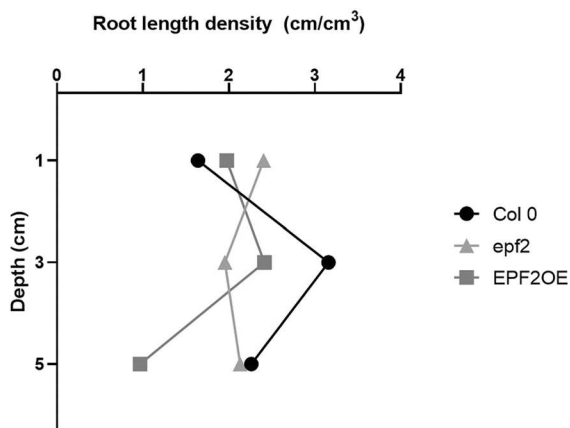
Fig. 8 Variation of root length density with depth in the different *Arabidopsis thaliana. L.* genotypes

As expected, stomatal density in the EPF2OE lines with increased expression of the *EPF2* gene had severely reduced SD as compared to the wild type. This is similar to what was observed by Hepworth et al. (2015) and Lundgren et al. (2019) who reported a SD reduction of between 60 and 65% in the same genotype as compared to a 73% difference in this case. On the other hand, the SD of the *epf2-1* mutant was 44% higher than that of the wild type which was relatively lower than that reported by Hunt and Gray (2009) who reported a 70% increase in the same genotype. The differences in SD in this study as compared to previous studies may have been due to the use of different growth media (mineral soil) used in this study instead of compost. This change may have had the effect of altering root-soil interactions which could have affected leaf cell developmental expression thus altering SD to some extent.

In line with our SD results, the EPF2OE line showed significantly improved WUE as compared to the wild type using both the Δ and biomass method of measuring WUE. This was probably as a result of reduced transpiration from the leaf surface which conserved water within the leaves. Also as anticipated, using the Δ method, the WUE of the *epf2-1*

mutant with increased stomatal density had significantly reduced WUE as a result of the increased SD. Surprisingly however, there was no significant difference in WUE between the wild type plants and the *epf2-1* as calculated from biomass and total water transpired. This could possibly be explained by the possible improved water conservation that could have occurred as the soil dried quicker when *epf2-1* plants were grown. This in turn may somewhat have induce a drought response possibly had the consequence of limiting transpiration to some degree.

Imaging of *Arabidopsis thaliana. L.* roots using NCT proved quite challenging as the size of the roots (≈ 80 µm) was too small to enable near complete root segmentation from the resolution we achieved (55 µm pixel). Also a delicate balance between amount of water applied and what would be optimal for NCT was difficult to achieve as *Arabidopsis thaliana. L.* roots dry quite rapidly as compared to other plants, limiting how dry the soil can be during imaging. Only the main taproot near the surface of the soil was visualised and thus we focused RSA assessments more on results of sX-Ray CT.

The sX-Ray CT scans revealed much greater detail of the *Arabidopsis thaliana. L.* roots as compared to previous studies using soil as a growth media e.g. Morris et al. (2017). The root properties indicated that there was generally no significant difference between the different root architectures of the three genotypes grown. This is contrary to what was found by Hepworth et al. (2016) who observed a generally reduced root growth in EPFOE mutants as compared to wild type plants. There was also no significant root growth difference between wild type and the *epf2-1* mutant with higher SD which was also contradictory to findings by Hepworth et al. (2016) albeit using a different genotype i.e. (*epf1epf2*) a double mutant with much higher SD in comparison. Although the differences that were shown in our study were insignificant, the EPF2OE mutant however tended to have the smallest RSA and reduced lateral root density which partially supports Hepworth et al. (2015)'s findings who postulated that the reduced transpiration tended to reduce root growth. Further analysis of root length with depth also revealed that the RLD of the EPF2OE tended to be lower at depth which partially explains its differences with the wild type plants in better detail. Root length for all genotypes in our experiment also tended to correlate well to shoot dry biomass suggesting that shoot biomass had a direct bearing on root growth.

Due to the nature of our experiment, it is worth noting that the potential differences which could have been observed in this study as opposed to other experiments using the same genotypes may, however, have been made moot by the non-uniform nutrient distribution within the soil used which may have influenced root growth. This is because in mineral soils, such as in this experiment, roots exhibit a greater degree of plasticity and thus variation in root architecture may be more difficult to definitively asses with relatively few replicates as in our case. The plants used in this study were also younger (26 days) as compared to those assessed in Hepworth et al. (2016) (35days) thus further complicating comparison between our experiments. Even though our experiments were conducted in a mineral soil, it is noteworthy however, that *Arabidopsis thaliana. L.* root growth in this experiment may differ from that under natural conditions (such as field conditions) as our soil packing and manipulation did not truly replicate field soil structure. Furthermore, roots in our experiment were also affected by the bonsai effect where they are restricted by the growth container.

In general, our results did not show any adverse effects associated with the WUE coffering EPF2 overexpression as in the EPF2OE mutant. This suggested that alteration in the EPF family of genes could be a suitable way of improving WUE without affecting root performance and thereby nutrient acquisition and thus enabling sustainable production of plants with altered stomatal development, though this would need to be experimentally determined for different crop species with larger root systems. In the context of climate change, while reducing stomatal density may lead to lower carbon utilisation and photosynthetic assimilation rates of plants grown in optimal conditions, in a world of increased freshwater scarcity, the increase in WUE makes reduced stomatal density a promising trait to help adapt to a changing climate.

Conclusion

This study reaffirmed the idea that improved WUE can be brought about by a reduction in stomatal density in well-watered conditions as shown in previous studies. We also showed that investigations on *Arabidopsis thaliana. L.* roots growing in soil can be successfully carried out using high resolution XRSI as there weren't

any major differences in root architectural properties between the genotypes. These results potentially indicate that cultivation of water use efficiency with alterations in the *EPF2* gene and hence stomatal density, does not impact soil properties. There is therefore the potential to generate more sustainable crops with improved WUE without any negative impacts on soil properties.

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