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Article

THROUGH THE LOOKING GLASS: Real time imaging in Brachypodium roots and osmotic stress analysis

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Abstract: To elucidate dynamic developmental processes in plants, live tissues and organs have to be visualized frequently and for long time periods. The development of roots is studied in depth at a cellular resolution not only to comprehend the basic processes fundamental to maintenance and pattern formation but also study stress tolerance adaptation in plants. Despite technological advancements, maintaining continuous access to samples and simultaneously preserving their morphological structures and physiological conditions without causing damage presents hindrances in the measurement, visualization and analyses of growing organs including plant roots. We propose a preliminary system which integrates the optical real-time visualization through light microscopy with a liquid culture which enables us to image at the tissue and cellular level horizontally growing Brachypodium roots every few minutes and up to 24 hours. We describe a simple setup which can be used to track the growth of the root as it grows including the root tip growth and osmotic stress dynamics. We demonstrate the system's capability to scale down the PEG-mediated osmotic stress analysis and collected data on gene expression under osmotic stress.

Keywords: Brachypodium, neutral red, root, Casparian bands, PEG-6000, osmotic stress, real time imaging, PDMS.

1. Introduction

The understanding of development in plants as well as morphological dynamics occurring under stress conditions in plants requires a broad quantitative analyses of the tissue and cellular dynamics [1–3]. The Brachypodium root has emerged as a feasible model system to study cereals organogenesis since grain grasses have either huge roots, e.g. maize, multiple roots, e.g. wheat or specialized water conditions e.g. rice [4]. It offers various advantages for live imaging such as its simple architecture with a single primary axile root until 3 leaf stage, its moderate transparency, small size and steady growth rate [5,6].

Techniques based on high resolution imaging and fluorescence microscopy with automation have increased considerably but have faced short time span limitations in the past [7]. Recently several new studies have emerged introducing specialized platforms, device systems [8] and microfluidic chips which reduce labor, cost, increase observation time and provide automated high resolution imaging. Relevant examples of developmental and reproductive organ analysis were followed for several days in some cases [9,10]. Many studies have taken into account plant and plantlike organs such as fungal hyphae, pollen tubes, and developing roots by high resolution microscopy and analysed physical and physiological dynamics [9,11,12].

While the analyses of several plant organs, organogenesis, pattern formation, growth dynamics have been observed in microenvironments the structures observed have been at the cellular level with few dicotyledonous species observed table 1. There has been little manipulation of monocot grain seedlings in such systems partly due to the size, scale and morphological intricacies of most grasses. Monocotyledonous seeds are usually elliptical, slender long grains, with embryo polarity which makes the germination behaviour at the tissue and cellular level distinct from dicotyledons.

For analyzing temporal changes occurring under osmotic pressure and to maintain continuous imaging data during the stress application in developing seedlings, long term analysis in a suitable setup is a requisite. To allow such imaging the specimen must be constantly under nutrient supply while simultaneously uninterruptedly accessible for visualization. While several studies of young Brachypodium roots have been carried out [5,13] the real time optical imaging of developing roots under stress has not been reported so far, due to the aforementioned technicalities.

Polydimethylsiloxane devices offer several advantages to create platforms for manipulating thick tissues to subsequently allow for live imaging of cellular dynamics. These platforms can be created into desirable designs, size and tailor-made structures for the sample under observation to allow high throughput imaging data. These PDMS chips can be made into high resolution microfluidic chips to visualize cellular dynamics or molded into larger organ-on-a-chip for tissue and plant-on-a-chip for whole plant growth analysis [12,14]. To capture long term adaptations of plant roots to different microenvironments several root chip microfluidic platforms have also been introduced.

We developed a simple and preliminary yet enlightening setup to visualize real-time live imaging of growing Brachypodium seedling root at tissue level in temporal resolution for 24 hours. By utilizing the architecture and size of the root the platform has been specifically developed to acquire stress adaptation information of the root with minimal apparatus complexity and nominal hardware, creating an inexpensive setup with off-the-shelf optical parts. Therefore, our work is comprised of a simple optical microscopy adjusted to a novel growth and continuously observable system which can follow root development and osmotic stress dynamics in a continually growing monocotyledonous root.

2. Results

2.1. Plant Growth in PDMS

PDMS strips with single, double and triple punches were tested for compatibility with Brachypodium seedling growth, presented in Fig.S1. Single, double and triple punch channels had volume capacities of 130, 280 and 385 µl, respectively. The growth compatibility and directionality observed in supplementary figure 1 C) provided initial data on how to handle the seed polarity in wells. Growth in was observed until the 3-leaf stage shown in E) which showed the plausibility of growing a grain seed relatively larger than Arabidopsis in a minimal quantity of growth media. In the 3-punch preliminary device with the 385 µl MS media capacity, five weeks of growth inside the Petri plate was achieved by refilling the wells with unsolidified agar every week. Growth was observed until the formation of a small adult plant (6 leaf stage) and this observation was comparable to the plant-on-a-chip setup, reported previously for Arabidopsis [9].

Figure 1 shows the basic design of a channel-based platform extrapolated from the initial seed growth observed in Fig S1. The inset shows the dimensions of the channels to ensure growth in a single plane of focus. B shows the actual plantlet growth inside the device placed in a tissue culture box to maintain humidity. The channels were flooded with liquid MS media instead of solid MS media as in Fig S1. In this apparatus the average plantlet growth was observed and compared to normal growth as can be seen in C. Despite the root growth being lower than normal growth due to constrictions in 3D space the growth of leaves was the same as that in normal and overall growth observed was in parallel to normal growth.

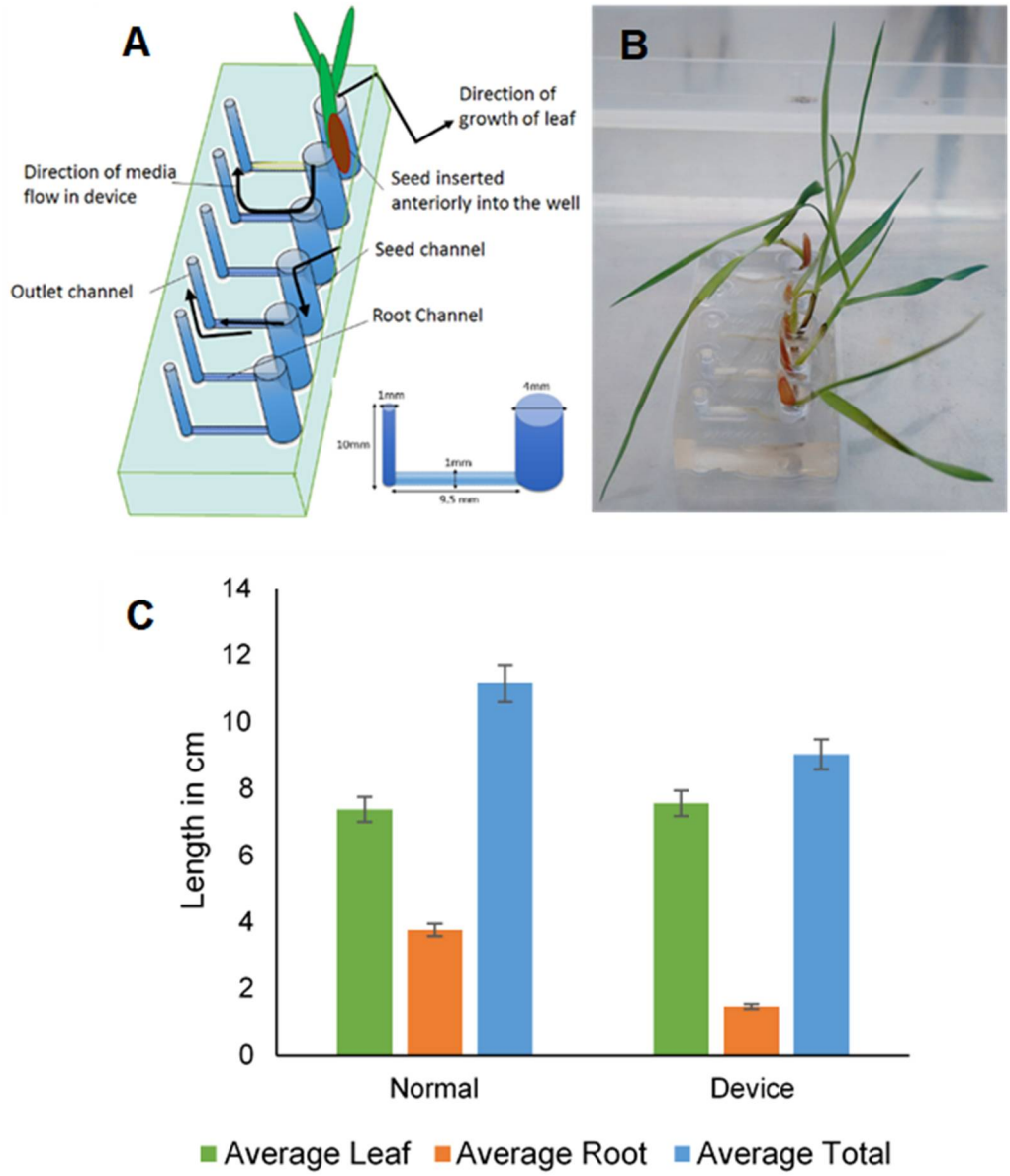

Figure 1. PDMS mold for growth and visualization analysis. The mold (A) used to construct the PDMS plant chip device (B) and comparison of the leaf and root growth in solid MS media plates and the plant chip device (C).

2.2. Visualization Setups

Figure 2 displays a detail of the images obtained from both the top and bottom imaging arrangements. In A) as mentioned before due to the size of the monocot seed more than 2 parallel experiments could not be observed simultaneously. However, the synchronous growth of 2 channels was analyzed in the top setting. In A) the axial rotation and the growth constraint of the ~450µm diameter root inside a 1000µm narrow channel can be visibly seen as curvatures and dents throughout the length of the root in the non-stained image and more accurately in the neutral red stained image. Fig. 2 B) shows the inverted fluorescent microscope focusing from the bottom directly onto the coverslip of the device. The bottom imaging setting allowed the imaging of a single channel at a time but nevertheless provided an accurate fluorescent signal for comparison of stress and control samples.

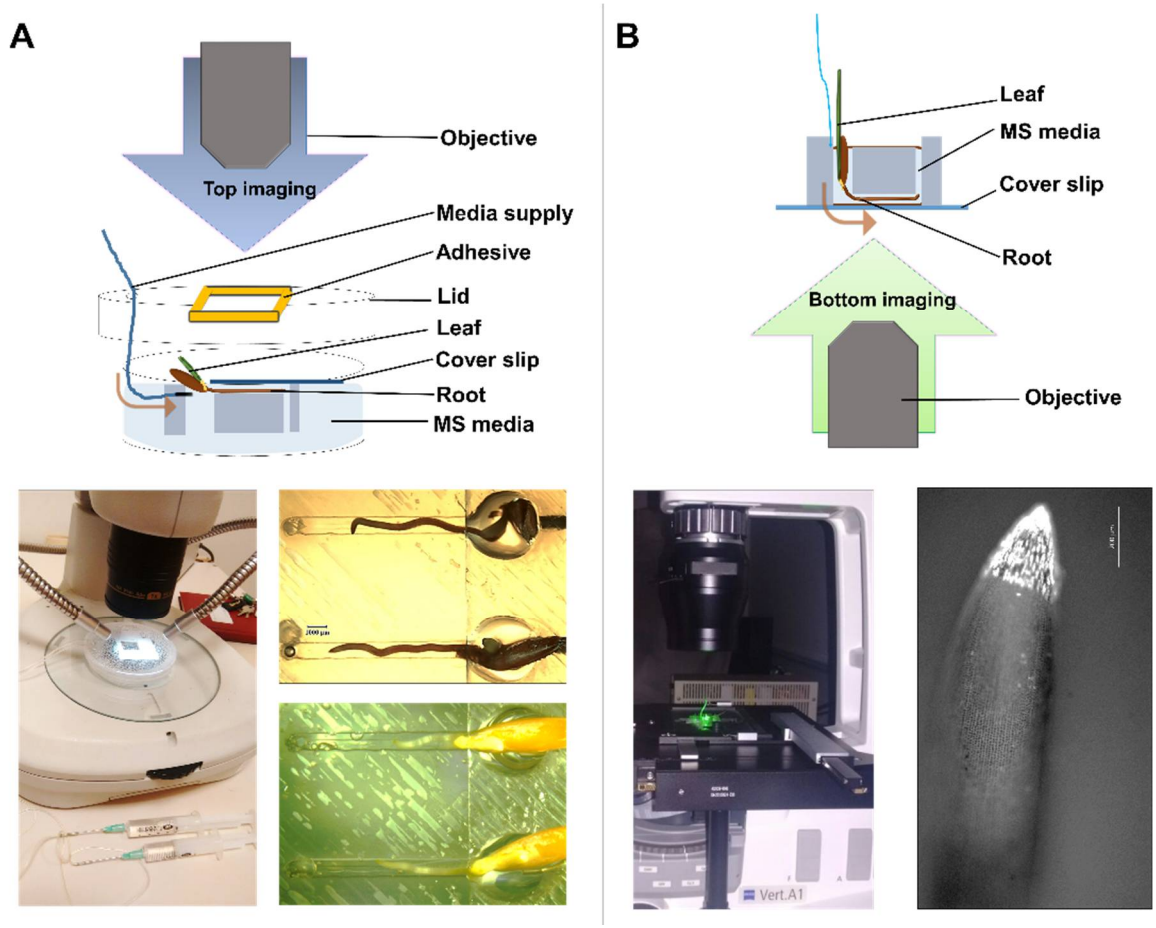

Figure 2. Experimental setups for imaging. Top imaging (A) and fluorescent bottom imaging (B). Three days-old seedlings having roots were mounted into wells for the top and bottom imaging. Top imaging studies were conducted with a Nikon SMZ 1500 stereomicroscope (Japan) while the fluorescent imaging studies were conducted with a Zeiss Axio Vert.A1 inverted microscope (Germany).

2.3. Time Lapse Growth Curve Analysis in normal conditions and Root growth under PEG

The growth rate of three independent monocot seedlings in the plant chip device under 16h day and 8h night conditions was observed with top imaging using the Nikon microscope Fig 3 A). The 24h time lapse video of Brachypodium seedlings was recorded at 24oC with a relative humidity of 37.5%. Brachypodium seedling roots showed slow growth with little change over time in the first 6 hours and then showed a slight increase in growth with a considerable increase in length during the night hours. This observation could be due to negative phototropism in roots. It was observed as a general trend that plant roots grow more rapidly in the dark hours as compared to the light hours as seen in the graph from time point 20.00-4.00. A slight surge in root growth was observed in the early morning hours followed again by a steady and continuous decrease. With time lapse recording, per minute and per hour growth was recorded and the growth over 24 hours was also monitored. In the plant chip device, the growth per minute was 4.3 μm min-1. The growth over a 24-hour period in Brachypodium showed a similar trend to that seen in Arabidopsis in previous studies (Grossman et al and Yazdanbakhsh et al). In the same setup 20% PEG-mediated osmotic stress was given and the growth of the roots for 12 hours were observed (Fig 3 B and C). A drop in the growth of roots was observed with almost continuous cessation of growth after 2-4 hours of osmotic stress as be downfall trend in growth in μm in B, and a constant root length in C. The length of both seedlings showed overall no growth under the osmotic stress. Since growth of the root showed a plateau after

approximately 4 hours, we choose 6 hours as a definite starting time point to observe stress under fluorescence for morphological changes in the root.

2.4. Root Cell Morphology and Horizontal Section Analysis

Normal growth in the plant chip device was maintained for 72h (3DAG) after which the root was subjected to stress. After 6 hours of stress the device was placed on the fluorescent microscope stage for bottom imaging (see Fig 2B). Fig. 4 shows the longitudinal section of the maturation (differentiation) zone. In the normal root plant, we can see the endodermal cells brightly stained with neutral red and the Casparian bands present. The root hairs and epidermal cells are not stained, and root hairs are dark extensions. In 6-hour, drought conditions we can see immediately the regulation of water intake by the Casparian bands and their rigid square formation over the length of the root cells. These results are interesting as the vivid Casparian bands depict increased suberization for increased water retention which causes them to be sharply visible under osmotic stress, the cell walls of the endodermis cells are vaguely visible underneath the Casparian bands as is shown by the blue arrow (D).

The Casparian bands prevent the high osmotic stress and negative water potential created by PEG-6000 to cause leakage of water from the stele figure B. Also, the growth of several lateral roots was observed in the stressed samples, indicating an adaptive behaviour of the cells to expand the space and surface area for further water uptake [15]. This behaviour of root hairs showing extensive growth was also observed after 18-hour osmotic stress on the root tips (Fig. 6 A and B). To confirm that the boundaries observed were of Casparian bands which became compact and prominent around shrunken endodermal cells we observed the cells in a higher magnification after 24-hour stress (E & F). In the normal sample vacuoles were observed clearly in the root endodermal cells shown with arrow in E). In the 24-hour drought sample, the fluorescence signal was minimal with no vacuoles visible, and the staining of the Casparian bands was not visible. Vague zones of fluorescence were observed (F, arrow), which could be lignin and suberin accumulation in Casparian bands at 24 hours. Limited data could be acquired from the longitudinal section of the root after 24 hours in 40x magnification thus to have a more precise and comprehensive view of suberin accumulation and cell morphology after 24-hour stress; we proceeded to prepare cross sections to understand which cells showed fluorescence after osmotic pressure.

2.5. Cross Section Analysis

The cross sections of various zones across differentiation and elongation zones showed that the endodermis was stained and not the epidermis. This confirms the observation in Figure 4 that the compartments analyzed were of the casparian tubes of the endodermis and not the epidermis. Figure 5 shows the cross section of the Brachypodium root under unstressed and osmotic stress conditions after 24 hours. The most prominent changes can be observed in the root differentiation zone A-B and in the root elongation zone G-H). In the maturation zone, the vascular bundle shows visible shrivelling of their cells though the shrinking is more visible in B) as compared to D) and F). The metaxylen and protoxylem in A) appear larger and protruding. The endodermis desiccated around its periphery. A similar appearance of dehydrated cells was observed in D) and E) comparing to C) and E) The endodermis cells together with the vascular bundle and pith are condensed in D) and F). The zone of elongation with no clearly defined vessel cells. Also, there was no suberin or lignin increase observed in the epidermis since those zones remained unstained under PEG-mediated drought conditions. At the beginning of the elongation zone the fluorescence was considerably reduced showing that the place where the first sieve elements start to differentiate were affected by drought Fig 5 H). Overall the cross sections reveal abnormal morphology within the stele region of the sample under 24h osmotic stress induced by PEG. The morphology of the midsection of the root was also analyzed with and without fluorescence as seen in Supplementary Fig. 2. The confocal microscopy results conformed to our observations in Fig 5B). Furthermore, the observation of the Casparian bands in Fig 4 B, D, F) was confirmed by the brightfield microscopy of the longitudinal

view of the stele. With neutral red the endodermis, stele was stained and not the outer part of the root or root hair.

2.6. Root tip analysis

In accordance with these results, Fig. 6 shows images of elongation zone cells obtained from plants under normal (A and C) and 24h osmotic stress conditions (B and D). An abnormal differentiation of the root cap hair was observed under brightfield imaging 18 hours after osmotic stress. This adaptation could be to increase surface area for improved water absorption. It is interesting to note that this observation could not be recorded in root stained with neutral red under brightfield (data not shown). On the other hand, a high fluorescent signal with bright and distinctly visible vacuoles appears to be higher in the root cap cells under standard growth conditions (C and E).

Under osmotic stress no fluorescence was observed in the root cap cells —which are the first sites of the plant in direct contact with the osmotic stress induced by the PEG molecules— as can be seen in Fig. 6 (D and F). The cell division zone shows fluorescence showing that under stress the meristematic zones increases its activity. Since neutral red is a vital dye for staining dead/alive regions its can be enough to state that illuminated regions under PEG stress depict mitotically active cells as in D).

2.7. Plantlet Growth Maintenance

In Fig 7 we analyzed the maximum extent to which we could maintain the Brachypodium plantlets inside the artificial setup environment. The graph shows the average growth of the plant's roots and shoots obtained from the array obtained from 26 seedlings after 1-month growth in PDMS, however extensive browning was observed at this stage thus we propose to analyze healthy green plantlets the experiment should not last more than 3 weeks. The average height of the leaf was recorded as 13 cm, average root length 1.63 cm, maximum shoot length of 22.5 cm and root length of 2.6 cm was obtained after 4 weeks growth, which we propose as the maximum period to maintain the Brachypodium seedlings in the device (Fig. 7). Our purpose was to approach the maximum limit until the leaf senescence would start to manifest. The apparatus gives the potential of a plant-on-a-chip device. Furthermore, enough downstream applications such as stress application and RNA isolation for gene expression analysis.

2.8. Gene Expression Analysis

In order to have a broad idea of the plausibility of utilizing this artificial setup for genetic stress analysis several different genes were selected, 2 genes which are well established as upregulated, one was a predicted gene not experimentally validated, and 2 genes that were downregulated. No clear trend was observed at short term osmotic stress over a period of 2 hours. Since the expression pattern obtained for each gene was not definitive, we decided to proceed with greater osmotic stress over a longer period. 20% PEG was applied over a length of 24 hours. The data obtained were in line with previous expression profiles of the selected genes.

With more osmotic stress 20% PEG and longer stress duration (4, 6, 8, 12, 24 hours) the expression profile of upregulated and downregulated genes under water stress was similar to that in previous data. BdNAC54 and BdNAC92 were downregulated as has been previously reported [16]. BdLEA5 is a gene upregulated in developing stages in plants, so its high expression was expected. DREB is a well-established drought responsive gene [17,18] and was upregulated at 4 hours and then displayed a 1.5 fold decrease at 6 hours and remained more or less the same until 24 hours. The most interesting observation was of the BdDi19 gene which is reported here the first time in Brachypodium distachyon. Its expression increased 120-fold after 24 hours of osmotic stress as seen in Fig 8. Dehydration-Induced 19 protein is a characterized protein in Oryza sativa having conserved domains in most grass species including Aegilops tauschii, Hordeum vulgare, Setaria italica among many

other Poaceae. This is the first time it has been experimentally verified to respond to drought in any plant species.

3. Discussion

Growth, directionality and compatibility was observed for Brachypodium seeds on all three PDMS punched molds and the results were in line with the previous reports conducted with Nicotiana and Arabidopsis [14,19,20]. After several experiments, we concluded that after 4 days of vernalization and 2 DAG seedling stage, the seedling had to be inserted in the correct orientation in the chip to make it grow along the length of the narrow 1mm channel. Growth was observed with the root penetrating the length of the microchannel with a slight curvature and bending.

A study on young wheat seedlings shows cell wall expansion in the maturation zone upon a low water potential around the roots and the authors suggest the accumulation of some solutes within the elongation and maturation zones in order to maintain the turgor pressure, resulting in an increase in the root diameter [21]. Although not seen in maturation zone cells, but a similar swelling behaviour of cells at the root apical meristem zone upon treatment with 5% PEG was previously reported for Brachypodium as well as wheat, rice, soybean, and maize [22], suggesting a collective response by root tissues of different plants to surmount the osmotic stress. The observation of Casparian strips in the endodermis is in line with results on waterlogging in Brachypodium since both stagnant water and dehydration with PEG are osmotic pressures on the cells [13].

Our results show the ease of visualization and utilization of neutral red as a promising dye for monocot PEG mediated stress without tissue processing. Since Neutral red stains casparian tubes it is an excellent vital stain to be used in monocots for in situ analysis without processing the tissue or sample preparation.

The Di19 protein was first reported in Oryza sativa as OsDi19-1 through RNA-Seq data. In Triticum aestivum it was known to have a C terminal domain. At the N terminus is a zinc finger zf-Di19. It was reported to be involved in environmental stress i.e. cold, drought, osmotic stress, and salinity. The protein was induced by high levels of abscisic acid and ethylene [23]. Though spatio temporal expression analysis the stem root and leaf overall showed an increase in expression of OsDi19 as compared to all other tissues (http://ricexpro.dna.affrc.go.jp/GGEP/graph-view.php?featurenum=12093#tabs-2). Under flooding and osmotic pressure, the expression also increased compared to other stress conditions such as salinity, cold, dryness, cadmium and hormones (https://tenor.dna.affrc.go.jp/EPV/Os05t0562200-01/). BdDi19 is thus a little-known gene which has a profound expression during short term osmotic stress in young seedlings. Further studies on this gene and protein in Poaceae could be useful in understanding dehydration and osmotic stress in relation to development.

This is the first study to incorporate neutral red for Brachypodium morphological analysis; this is also the first study to analyse PEG-mediated stress with neutral red dye. Moreover, this is the first study to experimentally validate Dehydration Induced 19 protein and analyse the expression levels of BdDi19 under drought stress.

We propose that a follow up study on Brachypodium seedlings in automated microfluidics or bioMEMS devices can be built upon our observations. Organ growth dynamics, root elasticity, microfluidic flow analysis and root hair dynamics under real time are a few of several areas which are desirable to be undertaken to unravel yet unknown physical patterns of growth and growth cessation and adaptation in favorable and stresses conditions.

4. Materials and Methods

4.1. Device fabrication

Rectangular PDMS pieces with a scale of 65x20x10 mm single, double and triple punched with 5 mm diameter punchers were initial seed growing reservoirs at different volumes to check biocompatibility. Acetone cleaned glass slides and PDMS pieces were plasma treated and bonded to get the final devices, which were used to test the compatibility of Brachypodium seeds with PDMS.

A mold for the plant chip was designed with SOLIDWORKS Software, reproduced onto ABS 3D material, and 3D printed. The mold dimensions were10mm height, 9.5mm channel length, 1mm outlet diameter, and each seed channel 4 mm in diameter. The channel height was fixed at 1 mm to ensure the growth of the root to remain in one plane and not be out of focus in the Z-axis under microscopy as was earlier observed for 2mm channel width and height in Fig. 1.12. For the construction of the device, PDMS and curing agent were mixed in 10:1 ratio and poured into the mold in a 100-mm diameter Petri dish, degassed in a desecrator, and cured at 75 °C for 60 min in an oven. The PDMS pieces were cut and gently peeled off from the mold on the Petri dish. The constructed device was submerged in Murashige and Skoog media overnight to ensure the hardening of the device. 0.17 mm coverslips and the PDMS pieces were plasma treated and bonded to get the final devices. Coverslips were used instead of the glass slides to facilitate fluorescent imaging. This setup was fixed with an adhesive to the Petri plate cover. Each channel was filled with MS media.

4.2. Plant Imaging and growth chamber

For each channel a seedling was grown in conventional petri plates with Murashige and Skoog solid media in closed and sterile conditions. The channel was designed to restrain the root growth to a horizontally narrow path (1mm diameter) which was optically transparent (0.17 mm glass coverslips). 4-days of vernalization and two days post-germination synchronously growing Brachypodium seedlings were inserted into the wells vertically at around 75-55° angle, with the scutellum facing slightly upwards and radicula facing downward to allow growth imaging of the roots in the horizontal narrow channels. The anterior end was immersed in the well, and the posterior end was entirely out of the well, with the emerging leaf facing outwards. This provided gas exchange and illumination for the leaves.

4.3. Seeds Preparation and Growth Measurement

Brachypodium wild-type seed line Bd21-3 was used in this study. The seeds were dehusked then soaked in water for 10 minutes. They were sterilized for 1 minute with 70% ethanol in a sterile Petri dish. Ethanol was drained, and the seeds were rinsed with sterile deionized water. 20 ml of 1.3% NaOCl solution was poured into the Petri dish and rotated for 5 minutes. The seeds were then rinsed thrice with sterile deionized water. Ten seeds were placed in between two layers of sterile filter papers soaked in sterile water. After 4-day vernalization, the seeds were transferred to agar media and allowed to grow for 48h at 22oC with a 16h photoperiod and high relative humidity at 57%. Finally, the seedlings were transferred to the device. Epson perfection v700 photo scanner was used to visualise the full length of the seedlings grown in the microfluidic device and standard agar environment. WinRHIZO software (Regent Instruments, QC, Canada) was used to analyse the shoot and the root scan images (Fig. 1.12).

4.4. Imaging Setups

Two different designs were developed for top and bottom imaging studies. The top imaging setup consisted of the same PDMS mold with the cover glass covering only the outlet channel, and the root channel, the seed channel was kept empty. This setup was then sealed with a double-sided adhesive tape to a carved-out Petri plate cover. The plate was filled with MS media and connected to syringes to ensure no air bubbles in the channels as well as constant media replenishment. For continuously monitored samples, the sample was illuminated from the above with a Nikon Fibre illuminator CFI-230 light source. The bottom setup consisted of the glass cover slip bonded to the entire device base to cover all the channels and the samples. Seeds were inserted anteriorly from the top with the root in the narrow horizontal channel. Fluorescent laser illuminated the sample from below through the glass coverslip.

4.5. Optical Apparatus

The seedlings were selected at 2 days after germination (DAG) for microscopy studies. For standard visualisation of the control and stress samples, the device setup for top imaging was used. PEG-supplemented MS media was used for osmotic stress. The top imaging was performed using Nikon SMZ 1500, Olympus SZ61 stereo microscopes and illuminator lamp Olympus LG-PS2 from Japan. For bottom imaging of the samples with fluorescence, a stock solution of 4 µM neutral red stain was prepared with 0.2X MS medium supplemented with 20mM potassium phosphate buffer at 8.0 pH, according to the procedure reported earlier [24]. The control and stressed plantlet roots were stained for 15-20 minutes following the removal of PEG-supplemented MS media. The staining procedure made the cells stained under brightfield and enabled fluorescent visualisation of the seedling roots. Cross section samples were prepared according to the protocol described online [25]. Fluorescence imaging was performed with Axio Vert.A1 inverted microscope by Carl Zeiss (Germany), using the bottom imaging setup. Confocal microscopy was performed with Carl Zeiss LSM 710, Germany and images recorded with Zen software (Carl Zeiss, Germany). Neutral red dye was used to visualize the live/dead parts of the roots of the young seedlings both for normal growth and for osmotic stress conditions. A single channel was used for visualisation with neutral red. Images were taken in 20X objective lens. Three-week old seedlings pre-stained with neutral red at the 2-DAG (days after germination) seedling stage (stained as mentioned previously) were selected. These seedlings were given osmotic stress for 6 hours in Murashige and Skoog (full strength) media with 20% polyethylene glycol 6000. Stressed and normal seedlings were embedded in agarose (as described for the fluorescence microscope staining) to enable section slicing as thin as possible. Cut sections ~ 0.5-0.9mm were achieved from the maturation zone of the plant. Transverse sections were removed from the agarose molds and placed separately on acetone-ethanol cleansed cover slips and glass slides. The cover slips were sealed securely with clear nail polish.

4.6. Imaging Acquisition

For continuous real time imaging acquisition the Nikon stereomicroscope SMZ 1500 was used together with the Nikon Fibre illuminator CFI-230 light source. Image acquisition was done with Spot Basic program. In the beginning of each experiment the root was positioned into focus. Samples were illuminated from above with 2 double arms and brightfield settings were used with 2 gain. Transmitted lower illumination was used for image capture in night hours. Imaging was carried out at 1X magnification due to the large size 420 µm diameter non-transparent root. Image resolution was 1000 pixels per inch, sensor pixel size was 7.4x7.4 µm, 68 sensor pixels were equal to 100µm. Imaging was set to occur every 5 minutes. The root rotation around all three axes during continuous growth was monitored by the stereomicroscope without need for repositioning or refocusing for 24 hours. The day and night hours were maintained strictly and manually. Image acquisition was done by Spot Basic software. For normal growth curve 288 images were recorded for 24 hours to generate a time lapse video S1 video. The growth curve was generated by measuring every 12th image with the measurement settings in Spot Basic 4.7. For stress conditions the program was set to capture the image after every hour uptil 18 hours. Zen Blue program was used to acquire fluorescent and confocal imaging with manual image acquisition. For still imaging Fig S1 Kameran software was used manually in junction with the Olympus SZ61 stereo microscopes and illuminator lamp Olympus LG-PS2 (Japan).

4.7. Osmotic Stress Application

To give osmotic stress 20% PEG 6000 was dissolved into the MS agar media and filled in the seed and root channel to three-week plantlets at the 3-leaf stage. For 6h and 24h osmotic stress analyses, the seedlings were first stained with neutral red for 20 minutes and then transferred to the microchannel device containing 20% PEG-MS and visualised under fluorescence microscope.

4.8. Imaging for Osmotic Stress

For visualisation of growth, the model PDMS device was used in both dorsal and ventral positions. Top imaging was achieved by plasma bonding the glass to the dorsal side, but only covering the root channel and the outlet channel, leaving the seed channel open for insertion, as can be seen in Fig. 2 A. Petri plate was used for maintaining humidity and growth in which the radicula was inserted into the channel, with the coleoptile facing upwards and outwards and a gap created in the lid to ensure growth for the shoot. The coverslip was attached to the lid with a strong double-sided adhesive. The objective was positioned to focus directly on the cover glass and gap. Two holes were bored inside the lid to insert the valves for constant media flow. This entire setup was prepared aseptically under laminar flow hood. However, the seed part for shoot growth was kept uncovered during the length of the experiment. Media was inserted into the dish and into the device wells with the metal heads bored into the seed channel to ensure full media flow. The Petri plate lid and the bottom part was covered with paraffin film to ensure high humidity. The device could be maintained in this manner for 48h. The fluorescent bottom imaging was done with the entire ventral side of the device oxygen plasma bound to a glass coverslip. Seeds were inserted into the device with the coleoptile and radicula facing outwards and the bottom objective directly visualised the roots. The roots were separately stained with Neutral Red dye according to the protocol by Dubrovsky et al. [24] and rinsed in MS media and the channels filled with non-stained full strength MS media to avoid background. For fluorescence imaging 0.4 µM neutral red solution and a 15-20 min incubation stained the roots sufficiently. 20% PEG was applied to full strength MS media for microscopic visualization of stress response morphological change of 2 DAG Brachypodium seedlings for 6, 18 and 24 hours.

4.9. RNA isolation and DNase treatment

RNA isolation was done from the whole plantlets with a Zymo research kit MiniPrep RNA isolation kit. Briefly the tissue was homogenized in trizol reagent and transferred to column tubes. Flow through was DNase treated in the column, followed by prep buffer and wash buffers with intermittent centrifugations. The final eluted RNA was 20ul. The concentration of RNA was verified by nanodrop spectrometer. A bleach gel was used to analyse the integrity of the RNA. A 1% bleach gel was prepared in 1x TBE. 10X RNA loading buffer was used and a low molecular weight RNA ladder was used.

4.10. Gene identification and primer design

From the plant genome database (http://www.plantgdb.org/prj/GenomeBrowser/) the Brachypodium genome was used to search for upregulated genes in drought. drought responsive family protein 19 (now renamed Dehydration Induced19) DI19, Late Embryogenesis abundant protein Lea5, sequences were downloaded, and primers designed. Primers for DREB2A were taken from Feng et al 2015. Two downregulated genes BdNAC054 and BdNAC092 were selected and primers taken from You et al 2015. Ubiquitin BdUBC18 was taken as internal Control and the primers used for it were also from You et al 2015. Primers used are listed in Supplementary Table 1.

4.11. qRT-PCR

After confirmation of the integrity of the RNA samples, cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed by Light Cycler 480 System (Roche) using Perfecta SYBR Green Super Mix from Quanta. Amplification was performed in a total reaction volume of 10 µl containing 5 µl of 1X SYBR Green Super Mix, 300nM of each primer, and 100 ng of the template cDNA. The PCR thermal cycling parameters were set at 95oC for 10 minutes to activate the SYBR green followed by 40 cycles of 95 oC for 15 seconds and 60oC for 1 minute. For each sample, three technical replicates were made. Plant samples without stress treatment (0 hour) was used for normalization and fold change calculation. The ΔΔCt Pfaffel method was used to analyze the relative gene expression of the qPCR results.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: HB, conceived the study. HB, ZK and MY designed of the study and drafted manuscript. HK designed the PDMS channel device. ME helped designing PDMS device. ZK carried out the all the experiments, analyses.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nezhad, A.S.; Packirisamy, M.; Bhat, R.; Geitmann, A. In Vitro Study of Oscillatory Growth Dynamics of Camellia Pollen Tubes in Microfluidic Environment. *IEEE Trans. Biomed. Eng.* **2013**, *60*, 3185–3193, doi:10.1109/TBME.2013.2270914.

2. Gooh, K.; Ueda, M.; Aruga, K.; Park, J.; Arata, H.; Higashiyama, T.; Kurihara, D. Live-Cell Imaging and Optical Manipulation of Arabidopsis Early Embryogenesis. *Dev. Cell* **2015**, *34*, 242–251, doi:10.1016/j.devcel.2015.06.008.

3. Bennett, M.R.; Hasty, J. Microfluidic devices for measuring gene network dynamics in single cells. *Nat. Rev. Genet.* **2009**, *10*, 628–638, doi:10.1038/nrg2625.

4. Brkljacic, J.; Grotewold, E.; Scholl, R.; Mockler, T.; Garvin, D.F.; Vain, P.; Brutnell, T.; Sibout, R.; Bevan, M.; Budak, H.; et al. Brachypodium as a Model for the Grasses: Today and the Future. *PLANT Physiol.* **2011**, *157*, 3–13, doi:10.1104/pp.111.179531.

5. Hardtke, C.S.; Pacheco-Villalobos, D. The Brachypodium distachyon Root System: A Tractable Model to Investigate Grass Roots. In *Genetics and Genomics of Brachypodium*; Vogel, J.P., Ed.; Springer International Publishing: Cham, 2016; pp. 245–258 ISBN 978-3-319-26944-3.

6. Girin, T.; David, L.C.; Chardin, C.; Sibout, R.; Krapp, A.; Ferrario-Méry, S.; Daniel-Vedele, F. Brachypodium: a promising hub between model species and cereals. *J. Exp. Bot.* **2014**, *65*, 5683–5696, doi:10.1093/jxb/eru376.

7. Massalha, H.; Korenblum, E.; Malitsky, S.; Shapiro, O.H.; Aharoni, A. Live imaging of root–bacteria interactions in a microfluidics setup. *Proc. Natl. Acad. Sci.* **2017**, *114*, 4549–4554, doi:10.1073/pnas.1618584114.

8. Sena, G.; Frentz, Z.; Birnbaum, K.D.; Leibler, S. Quantitation of cellular dynamics in growing Arabidopsis roots with light sheet microscopy. *PLoS One* **2011**, *6*, 1–11, doi:10.1371/journal.pone.0021303.

9. Jiang, H.; Xu, Z.; Aluru, M.R.; Dong, L. Plant chip for high-throughput phenotyping of Arabidopsis. *Lab Chip* **2014**, *14*, 1281, doi:10.1039/c3lc51326b.

10. Busch, W.; Moore, B.T.; Martsberger, B.; Mace, D.L.; Twigg, R.W.; Jung, J.; Pruteanu-malinici, I.; Kennedy, S.J.; Gregory, K.; Nc, D. A microfluidic device and computational platform for high throughput live imaging of gene expression. *Nat. Methods* **2012**, *9*, 1101–1106, doi:10.1038/nmeth.2185.A.

11. Grossmann, G.; Guo, W.-J.W.-J.; Ehrhardt, D.W.; Frommer, W.B.; Sit, R. V; Quake, S.R.; Meier, M. The RootChip: an integrated microfluidic chip for plant science. *Plant Cell* **2011**, *23*, 4234–4240, doi:10.1105/tpc.111.092577.

12. Bascom, C.S.; Wu, S.-Z.; Nelson, K.; Oakey, J.; Bezanilla, M. Long-Term Growth of Moss in Microfluidic Devices Enables Subcellular Studies in Development. *Plant Physiol.* **2016**, *172*, 28–37, doi:10.1104/pp.16.00879.

13. Shiono, K.; Yamada, S. Waterlogging tolerance and capacity for oxygen transport in Brachypodium distachyon (Bd21). *Plant Root* **2014**, *8*, 5–12, doi:10.3117/plantroot.8.5.

14. Meier, M.; Lucchetta, E.M.; Ismagilov, R.F. Chemical stimulation of the Arabidopsis thaliana root using multi-laminar flow on a microfluidic chip. *Lab Chip* **2010**, *10*, 2147, doi:10.1039/c004629a.

15. Paez-Garcia, A.; Motes, C.; Scheible, W.-R.; Chen, R.; Blancaflor, E.; Monteros, M. Root Traits and Phenotyping Strategies for Plant Improvement. *Plants* **2015**, *4*, 334–355, doi:10.3390/plants4020334.

16. You, J.; Zhang, L.; Song, B.; Qi, X.; Chan, Z. Systematic Analysis and Identification of Stress-Responsive Genes of the NAC Gene Family in Brachypodium distachyon. *PLoS One* **2015**, *10*, e0122027, doi:10.1371/journal.pone.0122027.

17. Hackenberg, M.; Gustafson, P.; Langridge, P.; Shi, B.J. Differential expression of microRNAs and other small RNAs in barley between water and drought conditions. *Plant Biotechnol. J.* **2015**, *13*, 2–13, doi:10.1111/pbi.12220.

18. Jiang, Y.; Wang, X.; Yu, X.; Zhao, X.; Luo, N.; Pei, Z.; Liu, H.; Garvin, D.F. Quantitative Trait Loci Associated with Drought Tolerance in Brachypodium distachyon. *Front. Plant Sci.* **2017**, *8*, 1–11, doi:10.3389/fpls.2017.00811.

19. Lei, R.; Qiao, W.; Hu, F.; Jiang, H.; Zhu, S. A simple and effective method to encapsulate tobacco mesophyll protoplasts to maintain cell viability. *MethodsX* **2015**, *2*, 24–32, doi:10.1016/j.mex.2014.11.004.

20. Ko, J.-M.; Ju, J.; Lee, S.; Cha, H.-C. Tobacco protoplast culture in a polydimethylsiloxane-based microfluidic channel. *Protoplasma* **2006**, *227*, 237–240, doi:10.1007/s00709-005-0142-2.

21. Akmal, M.; Hirasawa, T. Growth responses of seminal roots of wheat seedlings to a reduction in the water potential of vermiculite. *Plant Soil* **2004**, *267*, 319–328, doi:10.1007/s11104-005-0138-x.

22. Ji, H.; Liu, L.; Li, K.; Xie, Q.; Wang, Z.; Zhao, X.; Li, X. PEG-mediated osmotic stress induces premature differentiation of the root apical meristem and outgrowth of lateral roots in wheat. *J. Exp. Bot.* **2014**, *65*, 4863–4872, doi:10.1093/jxb/eru255.

23. Li, S.; Xu, C.; Yang, Y.; Xia, G. Functional analysis of TaDi19A, a salt-responsive gene in wheat. *Plant. Cell Environ.* **2010**, *33*, 117–29, doi:10.1111/j.1365-3040.2009.02063.x.

24. Dubrovsky, J.G.; Guttenberger, M.; Saralegui, A.; Napsucialy-Mendivil, S.; Voigt, B.; Baluska, F.; Menzel, D. Neutral red as a probe for confocal laser scanning microscopy studies of plant roots. *Ann. Bot.* **2006**,

97, 1127–38, doi:10.1093/aob/mcl045.

25. Schiefelbein Lab. Rapid Preparation of Transverse Sections of Plant Roots | Schiefelbein Lab Available online: http://sites.lsa.umich.edu/schiefelbein-lab/rapid-preparation-of-transverse-sections-of-plant-roots/ (accessed on Apr 1, 2017).