NOVEL BI-DIRECTIONAL DUAL-FLOW-ROOTCHIP TO STUDY EFFECTS OF OSMOTIC STRESS ON CALCIUM SIGNALLING IN ARABIDOPSIS ROOTS

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ABSTRACT

Being able to detect and respond to abiotic and biotic stresses is fundamental for plant growth and survival. However, understanding of signal transduction within the root remains limited. To help shed light on these processes, we have developed a bidirectional-dual-flow-RootChip (bi-dfRC), which adds bidirectional stimulation to the existing asymmetric laminar flow root perfusion platforms. In this paper we show design, fabrication and characterisation of the bi-dfRC, as well as growth of wild-type and Ca^{2+} indicator (G-CaMP3) *Arabidopsis thaliana* plants on the platform. Applicability of the bi-dfRC is further demonstrated by probing the dynamic response of *Arabidopsis* roots to simulated drought stress effects via a fluorescent Ca^{2+} sensor in a variety of combinations and spatial orientations. The latter enables the tracking of growth, localisation, and quantity in response to bidirectional stimulation in real time at a cellular level.

KEYWORDS

Root Chip, Abiotic Stress, Salt Stress, Signal Transduction, Calcium Signalling, *Arabidopsis.*

INTRODUCTION

Plant growth and survival are fundamentally linked with the ability to detect and respond to abiotic and biotic stresses. Observations show that climate change negatively affects plant performance, while conversely promoting pathogen infection on a global scale [1]. Adverse changes in the external environment will continue to have unequivocal impacts towards global ecosystem resilience, food quality and yield.

Until recently, elucidating the localisation and dispersion of key secondary messengers underpinning signal transduction in plant roots responding to abiotic (non-living, i.e. fluctuations in climate) [2] and biotic (living, i.e. pathogens) [3] stressors has been profoundly limited. Plant growth, development and adaptation are the outcome of copious signalling processes within the plant, comparable to neurofibrillary system in humans [4]. Understanding how molecular signalling events relay environmental stress effects on varying plant tissues including stem, leaves and roots is essential for increasing stress resilience in cultivated plants and management of natural ecosystems. Calcium (Ca^{2+}) is a key messenger molecule in biology, shared between many organisms. In plants, Ca^{2+} signalling is known to modulate for example hormone release, stomatal movement, targeted cell death and gene expression [5]. Cell specific Ca^{2+} signal responses to such stressors have been tracked utilising fluorescent dyes and genetically encoded fluorescent indicators (GEFIs) [6].

Research has focused on the role of signalling molecules in the aerial parts of the plant (shoot, leaves), due to technical limitations. Moreover, mimicking complex soil conditions in which plant roots inhabit, while quantifying signal intensity in a controlled environment has posed a challenge. New approaches are required to apply controlled stress treatments in a site-specific manner at local root cells, while tracking high-resolution signal transduction with fluorescence microscopy. Consequently, understanding how signal transduction for plant defence operates via Ca^{2+} decoding and communication between differing cell types in the root is largely unclear, especially under natural conditions.

Using microfluidic technology, soil-on-a-chip type platforms [7] have achieved cultivation of soil, plant, and the complex interactions between the micro-environment on chip. This concept has further been extended in the dual-flow-RootChip (dfRC) [8] platform, which added environmental complexity by incorporating root growth through a hydroponic channel and fine control of uni-directional treatment application via adjacent inlet channels. The latter in particular limits existing dfRCs to the application of stress conditions through 2 inlets, adjoining to the root at the maturation zone (mature tissue) of the cortical cells. As such, sight-specific treatment application within varying locally stimulated tissues, and the resulting directional polar dispersion and cellular localisation of Ca^{2+}, as illustrated in (Fig. 1(a)), are yet to be observed.

In this paper we present the development of the first bidirectional quantitative root chip platform for bidirectional signal quantification. Identical in dimensions to existing dfRCs, the polydimethylsiloxane (PDMS) bi-dfRC reported here adds a second set of inlet/outlet channels at the base of the observation chamber (OC) (Fig. 1(b)). These additional treatment inlets/outlets allow for independent or dual/asymmetric chemical stimulation at either the maturation zone or the root tip (Fig. 1(a)). As demonstrated via Ca^{2+} signalling, use of this bidirectional stimulation will yield exciting new insights into plant signal transduction and facilitate a platform for future research on root and plant stress perception.

EXPERIMENTAL METHODS

Microfluidic Device Fabrication

Photolithography [9] was used to fabricate bi-dfRCs moulds (Fig. 1(c)). Patterns were designed in L-Edit (Mentor Graphics v2020.1) and transferred onto photo-

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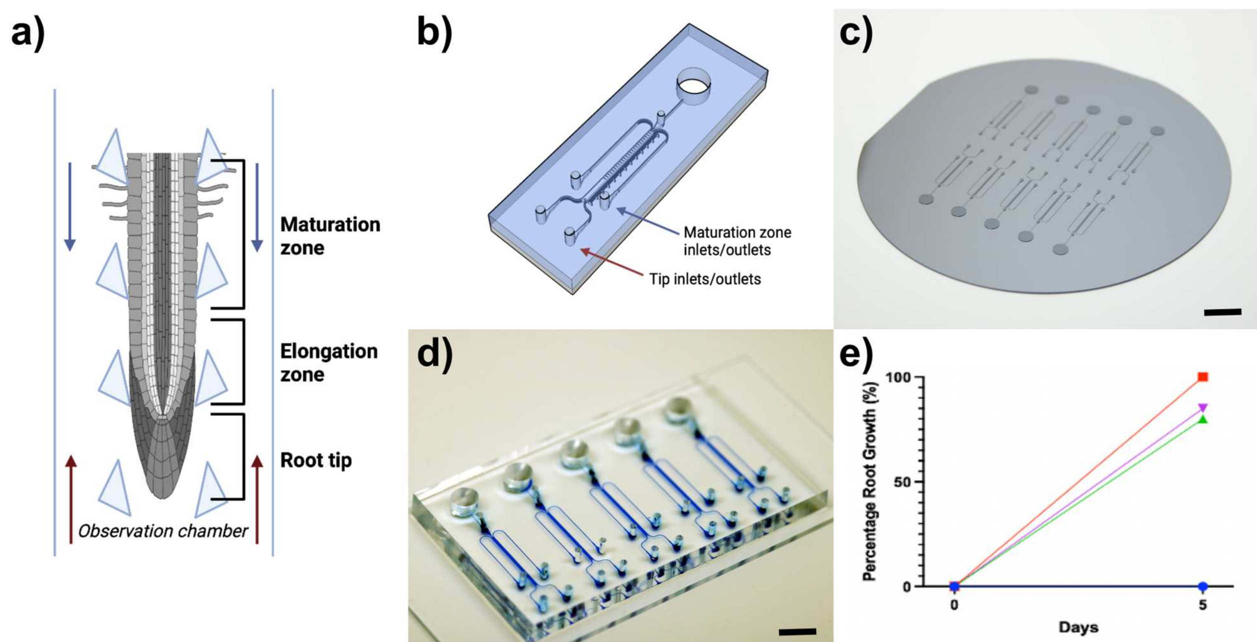

Figure 1: Bidirectional-dual-flow-RootChip design and fabrication. (a) Schematic diagram depicting a root cultured in the bi-dfRC microchannel with key root zones highlighted. Directional flow indicated by arrows (red; tip application, blue; maturation zone application). (b) Schematic indicating the additional tip inlets/outlets of the bi-dfRC. These are used as inlets during tip application and outlets during conventional maturation zone application. (c) Photograph of the silicon wafer mould with negative- tone dry-film photoresist defining 10 individual bi-dfRC devices. Scale bar = 12 mm. (d) Photograph of 5 bi-dfRC bonded to a glass substrate. Blue epoxy dye highlights the microchannels. Scale bar = 18 mm. (e) Line graph depicting total root growth (percentage; %) into untreated (blue dot; G-CaMP3 untreated, green triangle; Col0 untreated) and PVP-treated microchannels (red square; G-CaMP3 PVP treated, purple triangle; Col0 PVP treated).

masks (Nanofilm) using a laser mask writer (Heidelberg µPG101). Prior to cleaning via an O_2 plasma cleaner (PIE Scientific Tergo) for 10 minutes at 100 W, the wafer was dehydrated for 24 hours at 180°C. Next, lamination was undertaken to add a 100 µm thick negative-tone, dry-film photoresist layer (SUEX 100, DJMicrolaminates) onto the wafer. The bi-dfRC pattern was transferred into the photoresist via UV exposure in a mask aligner (MA-6, SUSS, MicroTec). A fitted dose of 166.6 mJ/cm^2 (365 nm) was applied. Next, the wafer was heated to 65°C on a hot plate for 5 minutes, then cycled to 95°C for 20 minutes, completing a post-exposure bake. The pattern was developed in propylene glycol methyl ether acetate (PGMEA) for 30 minutes. The transferred structures were then washed with isopropanol for 5 minutes and hard-baked at 125°C for 1 hour on a hot plate.

Replica moulding utilising pre-fabricated etched wafers was achieved through soft lithography [9]. Firstly, the silicon mould was treated with trichloro (1H,1H,2H,2H-perfluorooctyl) silane (Sigma) vapour to aid mould release. Polydimethylsiloxane (PDMS) pre-polymer silicone elastomer base (Sylgard 184, Electropar) was then combined at 10:1 (w/w) ratio with silicone elastomer curing agent. The mixture was degassed for 30 minutes to remove air bubbles. Next, PDMS was cast onto the wafer mould restricted by a poly(methyl methacrylate) (PMMA) ring and degassed for 1 hour. The set up was heated to 80°C for 2 hours on a hot plate to cure. Next, the PDMS was carefully peeled from the wafer mould and PMMA ring, followed by additional curing at 80°C for 2 hours. Next, inlet and outlet holes in the PDMS were created utilising 1 mm and 3 mm hole punches (ProSciTech) for treatment or root inlets and media ports, respectively. A guillotine was utilised to separate individual devices yielding 5 bi-dfRCs per substrate. In tandem, 24×60 mm glass microscope cover slides were washed in acetone, methanol, isopropanol for 5 minutes, respectively, under sonication. PDMS chips were then lightly pressed onto glass cover slips, bonding the exposed surfaces following O_2-plasma activation at 15 W power for 1 minute (Tergeo, PIE Scientific). Lastly, to strengthen the bond, chips were heated to 80°C for 2 hours on a hotplate. To visualize microchannels, Sudan dye (Sigma-Aldrich) was added to 1 mL toluene (Sigma-Aldrich) and Norland Optical Adhesive (NOA72, Norland Products) [10]. Following toluene evaporation the dye was passively injected into microchannels and cured using a spot UV curing system (OmniCure® S2000) (Fig. 1(d)).

Polyvidone Treatment

To reduce small molecule diffusion into PDMS and maintain hydrophilic retention of the bi-dfRC microchannels [11], chips were first exposed to 30 W power for 3 minutes in an O_2 plasma cleaner (Tergeo, PIE Scientific). Next, 22% w/v polyvinylpyrrolidone (PVP, Sigma-Aldrich) solution was passively injected into the chip microchannels for > 1 minute. Channels were washed 3-times with distilled water then desiccated and stored in vacuum-sealed bags.

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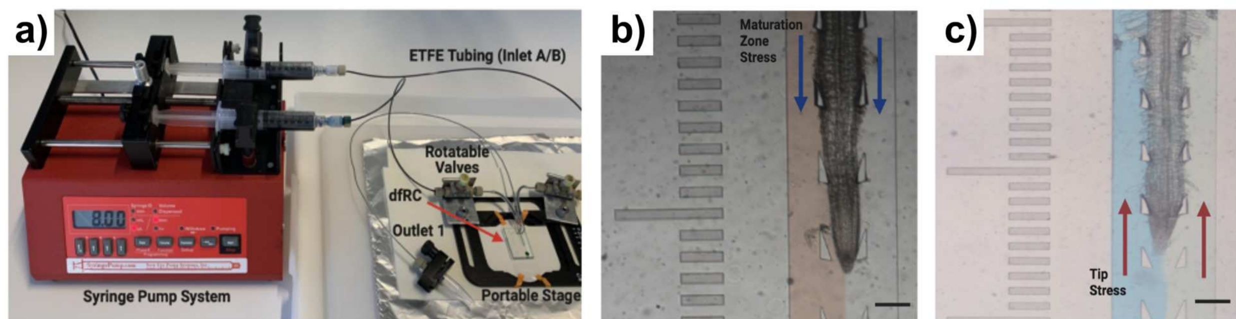

Figure 2: Asymmetric fluidic flow regulation, system setup and flow examples. (a) Photograph of the syringe pump system, tubing and chip adapter used for the delivery of asymmetric test solutions into the bi-dfRC microchannels. (b) Optical micrograph of asymmetric flow of test solutions (red and clear) from the maturation zone in the presence of wild-type Arabidopsis Col0 *root. Arrows indicate the flow direction. This flow condition is representative of that in conventional dfRC devices. (c) Optical micrographs of asymmetric flow of test solutions (blue and green) from the tip in the presence of* Arabidopsis Col0 *root. Flow remains laminar, as for the conventional dfRC case, and parallel flow streams split symmetrically on the root tip. (Zeiss 5x lens) scale bar = 300 µm.*

Imaging Set Up and Experimental Analysis

Identical in overall dimensions to existing dfRCs [8], the PDMS bi-dfRC reported here adds a second set of inlet/outlet channels at the bottom of the observation chamber (OC). These additional treatment inlets/outlets allow for independent or dual/asymmetric chemical stimulation at either the maturation zone or the root tip (Fig. 1(a)). Both, polyvinylpyrrolidone (PVP)-treated and untreated conventional dfRCs and bi-dfRCs were used (Fig. 1(e)). Wild-type and plant roots containing fluorescent indicator for Ca^{2+} (G-CaMP3) detection were left to grow into the OCs for 7 days, after which stress conditions were selectively applied at the root tip or maturation zone using an external fluidic setup (Fig. 2(a)). Ca^{2+} expression in roots was recorded on-chip using epi-fluorescence microscopy (Zeiss (AX10) 5× lens (EC Plan-Neofluar 5×/0.15 M27) plus eGFP filter (38 HE Green Fluorescent Protein BP 450-590). A dual-pressure syringe pump system (NE-1010, New Era Pump Systems Inc) connected to inlet channels via 1/16" OD ethylene tetrafluoroethylene (ETFE) tubing (Kinesis) provided flow control. The flow rates were set to 20 µL/min, fluorescent intensity was measured using ImageJ and data analysis was conducted in GraphPad Prism (version 9.2.0).

EXPERIMENTAL RESULTS

Asymmetric and Bidirectional Flow Capabilities

Fluid flow optimisation encompassed fine tuning flow rate control. By optimising tubing length and circuit components, backflow and leakage were limited. PDMS cracking at inlets was reduced by attaching flexible Masterflex Tygon Lab tubing (DO-06409-16; L= 1 cm) to stiffer ETFE tubing. Air bubbles were removed from microfluidic channels by pre-degassing to dry and removing gas from microchannels, pre-wetting before treatment application and/or generation of negative pressure via backflow for larger bubbles. Overall, flow rate control was tested in a bi-directional manner utilising coloured dyes, revealing steady asymmetric perfusion of test solutions in the presence and absence of a root and irrespective of the application direction (Fig. 2(b,c)). Dual-stream laminar flow was applied, as for tip stress conditions to observed reliably split symmetry on the root tip (Fig. 2(c)).

Hydrophilic Retention

After observation of limited root growth in naturally hydrophobic PDMS microchannels, these were rendered permanently hydrophilic to promote root elongation and growth on chip. Channels combining a hydrophobic surface of PDMS and hydrophilic surface of the glass cover slip base were clearly limiting the protrusion of drought and touch sensitive transgenic G-CaMP3 plant lines into the OC of the root chips, whereas wild type *Arabidopsis* roots showed no preference between untreated or treated channels (Fig. 1(e)). As shown in (Fig. 1(e)), Treatment of the bi-dfRC with PVP re-established 100% growth of G-CaMP3 into the root chips over 5 days of culture.

Root Ca^{2+} Localisation in Response to Salinity Stress

Wild type and G-CaMP3-modified *Arabidopsis* plants were successfully cultured on the bi-dfRC, then asymmetrically and bidirectionally perfused. Root growth on-chip showed a distinct preference for PVP-treated devices, indicating hydrophilic retention is essential for root growth in drought and touch sensitive G-CaMP3 Ca^{2+}-sensor plant lines, but not essential for wild type plant line Col0. Local and systemic cellular response of Ca^{2+} in *Arabidopsis* roots was characterized on-chip by measuring the fluorescent intensity at 5 linear sections within the tip/columella, elongation zone and maturation zone of the root. Ca^{2+} localisation rapidly increased within 5 seconds at the root tip and distal elongation and maturation zone following selective exposure of full burst $NaCl_2$ at the root tip (Fig. 3(a)). In contrast, full burst $NaCl_2$ at the maturation zone resulted in a fast Ca^{2+} upregulation which predominated within the epidermis and cortex of the elongation zone within 5 seconds following exposure (Fig. 3(b)). Nevertheless, the signal lasted longer when the root was exposed to the stress from the maturation zone. The appropriate signals then dissociated shoot or root-ward; away from initially stimulated cells in a systemic manner over 3 minutes.

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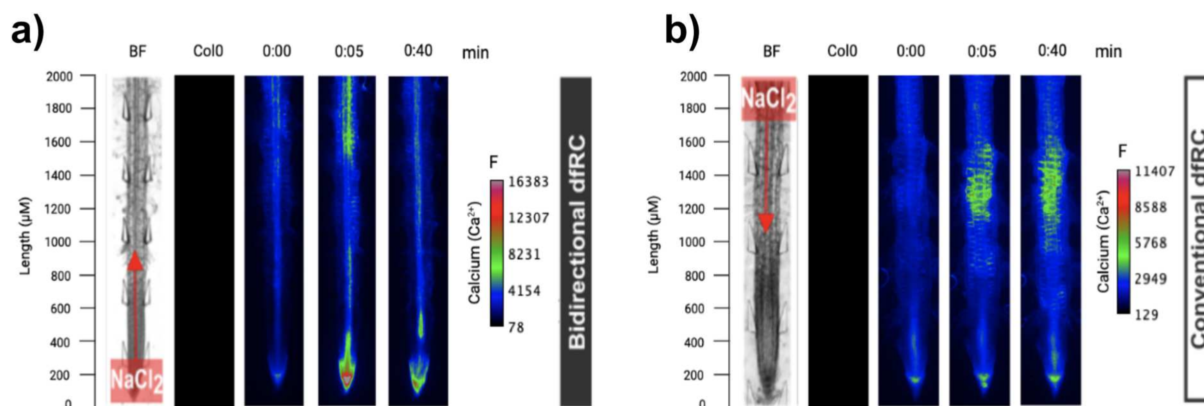

Figure 3: Ca^{2+} response to sodium chloride ($NaCl_2$) in 7 day old Arabidopsis G-CaMP3 *roots under epi-fluorescent microscopy. Images are depicted as heat maps (blue) (F= fluorescent intensity as gray value; pixel brightness). (a) Measurements of Ca^{2+} in response to targeted application of 100 mM $NaCl_2$ at the root tip zone as a heat map over time. (b) Measurements of Ca^{2+} in response to targeted application of 100 mM NaCl at the maturation zone as heat map. This example corresponds to the conventional dfRC use case. As can be observed, Ca^{2+} intensity, localisation and signal propagation differ from the root tip application, highlighting the importance of the bidirectionality of stimulus delivery. Bright field and Wild type Col0 are displayed on the left of each graph.*

CONCLUSION

In this paper we presented the development and application of a bidirectional root chip platform, which significantly extends the applicability of existing dfRCs for *Arabidopsis* root culture. The bi-dfRC incorporates 2 extra inlet/outlet channels for bi-directional stress treatment application at the maturation zone or tip of the root, while maintaining asymmetric fluidic flow control. In addition, surface area of the microchannels have been permanently altered utilising PVP treatment to accommodate drought and touch sensitive plant lines. Findings from this research reveal that the rapid Ca^{2+} burst localises at different root tissue in response to targeted application of $NaCl_2$-induced salt stress at the root tip or maturation zone.

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