Thesis Title:

Set up *Wolffia australiana* as a New Model Plant by Plant-on-chip System

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Abstract：

Wolffia is a small green aquatic plant that lacks differentiation of root, stem, and leaf, and produces new foliaceous individual on its branches. Over time, *Wolffia* has been considered an excellent subject of research in flowering physiology. Due to its simple morphology, easy cultivation, and growth and branching modes that differ from traditional model plants, *Wolffia* can be used as a new model organism with high value as an object of study of the core process of multicellular organism's morphogenesis. In order to achieve this goal, controlling the life cycle of *Wolffia* in the laboratory environment, especially inducing the transformation from vegetative growth to reproductive growth, has become the primary research task. For this paper, we first cultured the multicellutar organism *Wolffia australiana* in the microfluidic system and conducted morphological and physiological studies spherically. *Wolffia's* flower structure and branching process were observed and recorded by SEM. Using time-lapse photography using a Stereo Microscope, the life cycle of a single *Wolffia australiana was* tracked in detail.In addition, we induced *Wolffia australiana* to develop flower structures by a variety of reagent combinations, and found out the optimum concentration (10^{-4}M EDTA and 10^{-7}M SA) with the flowering proportion up to 25%. After obtaining flowering individuals of *Wolffia australiana* on the microflui platform (chip), we used RNA-seq technology to detect the differences in gene expression of different stages of *Wolffia australiana* flowering that under the induction of EDTA, laid a solid foundation for the subsequent analysis of the flowering regulation network. Through trace substance detection, we found that the concentration of metal ions in *Wolffia australiana* treated by EDTA changed and the content of Zn^{2+} decreased significantly. In conclusion, *Wolffia* combined with a "plant-on-chip" system can be used as a new model organism, addressing a lot fundamental questions which can not be answered using other model plants.

Keywords: model plant, *Wolffia* , microfluidics, plant-on-chip system,RNA-seq.

1. Background

Multicellular organisms can be classified as unitary organisms and clonal organisms. Since the 18th century, most studies on plant development have followed the study of animal development, with the idea of regarding the whole plant as an individual. In fact, the development of plants is obviously different from animals since most plants are clonal organisms, and are assembled from buds as developmental units[1].Hence, In order to better answer the question about the core process of plant development, a new model plant, which is simple enough to be classified as a unitary organism, conducting separate research using single buds, should be conducted. Fortunately, we found the simplest angiosperm, *Wolffia.*

Wolffia belongs to the class Monocotyledoneae, order Arales, family Lemnaceae and genus *Wolffia*. There are 11 species of the genus *Wolffia*[2], including the smallest angiosperm discovered so far. *Wolffia* is widely distributed in waters all over the world [2]. Each individual is a small green leaf-like plantlet, without differentiation in rhizome and leaves.

What makes *Wolffia* different is its unique way of branching, which *Wolffia* applies to reproduce. When a new branch is produced, it detaches from the previous branch. Each branch becomes two independent plantlets instead of growing into clumps as in most plants. The study of *Wolffia* is a study of a single plant development unit. Therefore, we believe that *Wolffia* can be used as a new model plant to answer the basic questions of plant development.

Wolffia has many applications, mainly focusing on feed exploitation, environmental monitoring, and sewage treatment, pharmaceutical product development, and other related fields. Its high quality protein content and balanced amino acid composition have high practical value in human nutrition [3].In addition, *Wolffia* is sensitive to the content of cadmium and lithium in the culture medium, so it can be used as a biosensor to detect water pollution [4]. It can absorb heavy metals, nitrogen, phosphorus and other pollutants within a certain limit, making it applicable to purifying water [5].

Studies on plants from family Lemnaceae were first reported in the late 19th century. German scientists Hegelmaier and Engler had an early understanding through research on the morphological structure and systematic classification of *Lemna*. Subsequently, the American scholar Florence and Indian scholar Sowjanja dove deeper in the structural anatomy and life history of *Wolffia* [6][7]. When it comes to flower induction, the Indian scholar Mahcshwari tried to ascertain the influence of salicylic acid, EDTA and zeatin in branching and flowering, and succeeded in inducing *Wo. Microscopica* to flower. Mahcshwari believes that *Wolffia* is an splendid subject for studying flowering physiology [8][9].

Wolffia australiana has the smallest genome of *Wolffia*. We contributed to the Genome sequencing and assembly of *Wolffia australiana* which will be completed

soon.In addition, the transgenic technology research on the *Wolffia australiana* is being accomplished by our cooperation units, the Aquatic Species Research Institute. The challenge of using *Wolffia as a model plant* is to control the life cycle of *Wolffia australiana* under laboratorial environments, especially to induce the *Wolffia australiana* from the vegetative growth stage to the reproductive growth stage.

In order to go through the complete life cycle of *Wolffia australiana* and accomplish the key step of establishing it as a new model, setting up an appropriate culture system to track the changes occurring in each developmental stage is essential. Traditional conical flask and petri dishes cannot effectively track and control individual *Wolffia australiana*, thus this experiment used microflow technology and tried to establish a "plant-on-chip" culture system in the laboratory to solve the problems that cannot be solved by traditional culture methods.

Microfluidic is a technology platform using the size of tens to hundreds of micrometers channel to change or control fluid and particles [11]. Since the 1990s, with the development of soft lithography technology, microfluidics has fully demonstrated its advantages such as small size, less sample required, easy to make and use, and its sensitive reaction, separation and detection [12]. The microfluidic platform is widely used in various biological fields, for instance in high-throughput drug screening, molecular sample preparation, reaction, isolation, detection and cell culture, sorting, cleavage, biosensor and instant diagnosis, etc. [13].

Due to the small size of *Wolffia australiana* (less than 1000 microns in length and about 600 microns in width) and the relatively short time of branching, *Wolffia australiana* can be used advantageously in the microfluidics, such as high throughput, easy control and accurate positioning to track the life cycle of individual *Wolffia australiana*, establish the new "plant-on-chip" culture system of *Wolffia australiana*, and complete its life cycle.

2. Results

2.1Morphological observation of *Wolffia australiana*

Wolffia australiana is a small plantlet with a swollen leaf-like shape characterized with dorsal-ventral polarity. The dorsal side (i.e.back side) which is exposed to the air is flat, with green color and certain number of stomatas. The ventral surface (i.e. abdomen side) which is submerged in the water is protruding downward with transparent and yellowish-green color (Figure 1). During vegetative growth, new individuals are produced by branching. Once the newly generated plantlets grow to a certain size, they detach from the original one (Figure 12). In the reproductive growth stage, the dorsal surface of *Wolffia australiana* is splitted. The flower organs extend out of a hollow hole (Figure 2). The flower are extremely simplified, without stalk, receptor and perianth. Only mono-stamen and mono-pistils are visible (Figure 3-4). Occasionally two stamens or two pistils may appear. The stamen anther of *Wolffia australiana* splits longitudinally, which has a single pistil, an ovary 1-locular, and a basal placenta [10].

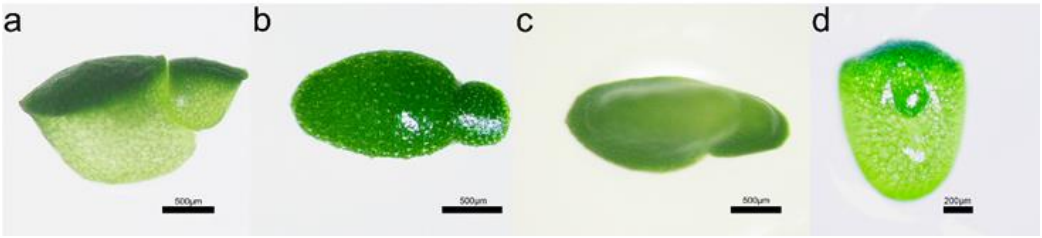

Figure 1 Growth process of Wolffia australiana

A Wolffia australiana with branching, a,b,c,d is the lateral, dorsal, ventral, and underside of the Wolffia australiana

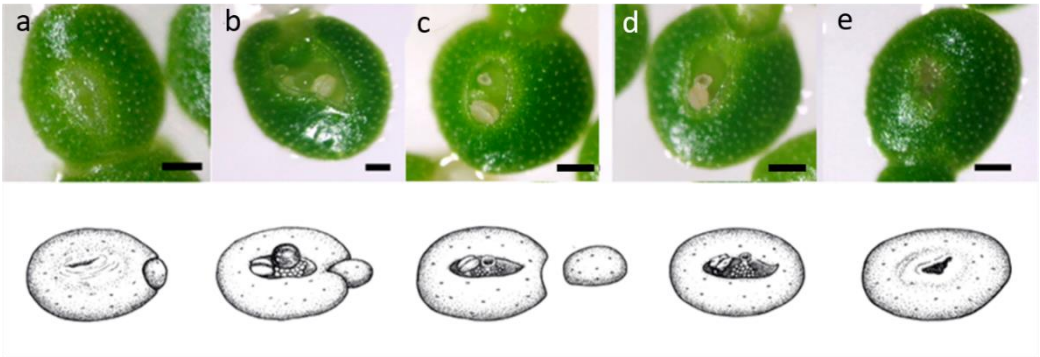

Figure 2 The flower process of the Wolffia australiana

a-e refers to the flowering process of a single Wolffia australiana plantlet, corresponds to the painting below (a)There are visible white areas (or cracks) on the center of the upper surface of Wolffia australiana. (b)Exserted pistil with droplet exudates. (c)The visible immature stamens. (d)The droplet on the pistil surface disappears and the stamen becomes pale yellow. (e) Pistil and stamen all become yellow brown, crinkle, withered. The pistil and stamen wither completely and the rift begins to heal.

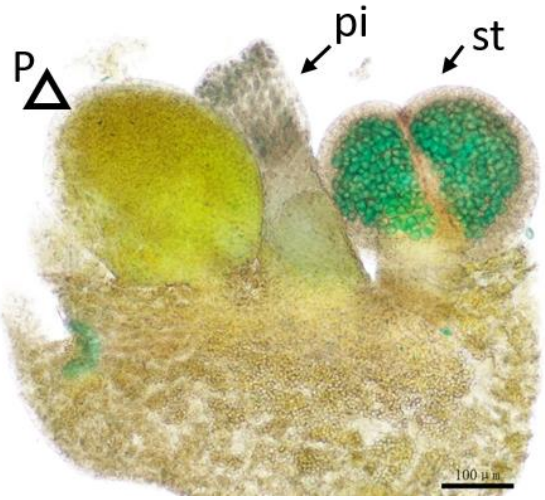

Figure 3 The flower organs of the Wolffia australiana

After transparent treatment, St was is the stamen (the green part was the stained pollen grains), Pi is the pistil, and P is the new branch (the yellow part).

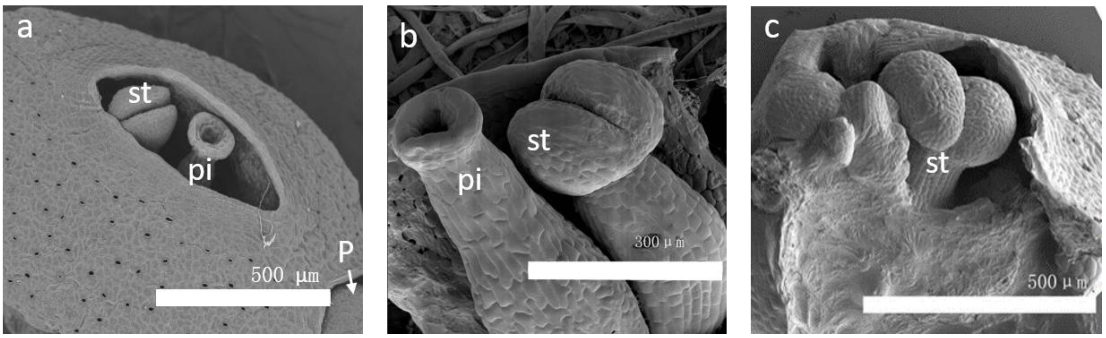

Figure 4 The flower organs of the Wolffia australiana

(a) the stamen developed medially relative to new plantlet, while the pistil lateral to new branches (b)(c) The stamens and pistil of a dissected plantlet. Stamen(st),pistil(pi),plantlet(P). Bar as indicated.

2.2 The Flowering Number and Branching Number of *Wolffia australiana* Induced by Alone Reagents

2.2.1 The Flowering Number and Branching Number of *Wolffia australiana* Induced by EDTA

EDTA (Ethylene Diamine Tetraacetic Acid) is a common chelating agent, which can chelate Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and other bivalent metal ions to form a stable chelate polymer. It can be used in plant studies to eliminate the inhibition in enzyme-catalyzed reactions caused by trace heavy metals.EDTA can induce *Wolffia microscopica* to flower, probably because of some of the EDTA chelation key metal ions, such as Cu^{2+} [14].

In the laboratory, *Wolffia australiana* was grown in 1/2 MS medium and did not flower without adding any reagents. After adding EDTA with a concentration gradient of 10^{-3}M~10^{-8}M, the growth rate of other was basically consistent with that of the control group,except the concentration of 10^{-3}M significantly inhibited its growth rate (Figure 5-a). After 5 days of EDTA treatment, there were plantlets flowered (Figure 5-b). We calculated the flowering ratio, but since the flowering ratio is greatly affected by the plantlet number, some concentrations inhibit the growth of *Wolffia australiana* but can also induce *Wolffia australiana* to flower, so the flowering ratio is not representative.Based on the growth and flowering of *Wolffia australiana*, 10^{-4} M EDTA was considered as the optimal induction concentration in this treatment.

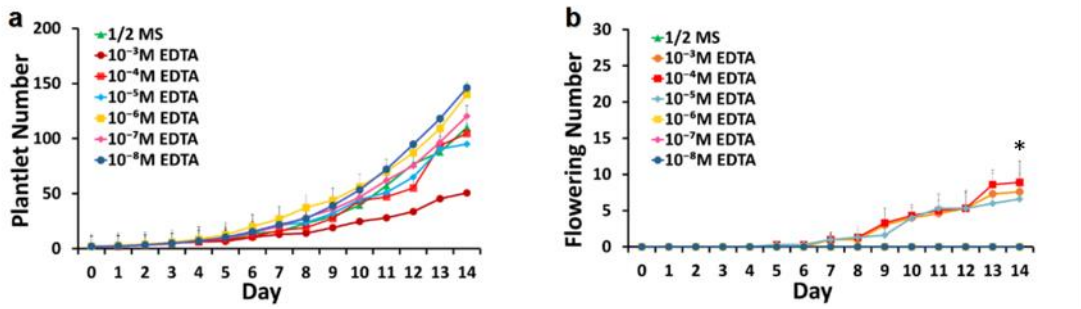

Figure 5 The plantlet number(a)、flowering number(b)of Wolffia australiana under EDTA floral induction

2.2.2 The Flowering Number and Branching Number of *Wolffia australiana* Induced by SA

SA (Salicylic acid) plays an important regulatory role in plant response to drought, cold, heavy metal poisoning, high temperature and abiotic stress [15].It is the most commonly used reagent to induce the flowering of various species of the *Wolffia* family, with relatively high induction frequency [16]. SA can also regulate the flowering of other plants [17].

In the experiment, the concentration of SA treatment ranged from 10^{-5} M to 10^{-9} M. The results indicated a very obvious inhibitory effect of SA on the growth of *Wolffia australiana* (Figure 6-a), but the number of flowers was increased, and the average flowering number of 10^{-5} SA floral induction could reach 10 in 14 days.(Figure 6-b).

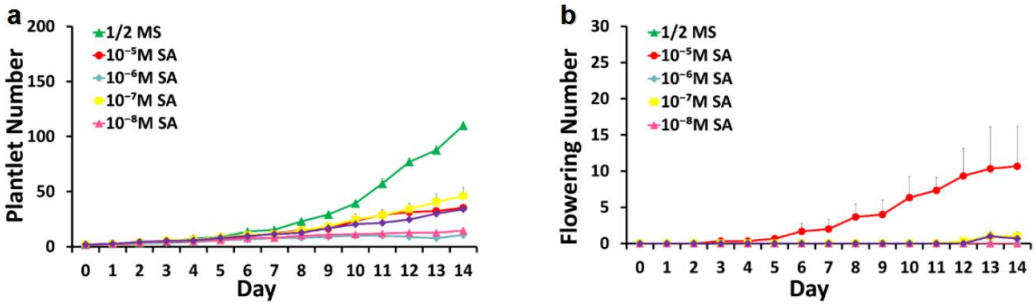

Figure 6 The plantlet number(a)、flowering Number(b)of Wolffia australiana under SA floral induction

2.2.3 The Flowering Number and Branching Number of *Wolffia australiana* Induced by GA_3

GA (Gibberellin) can promote plant flowering by removing the inhibition of key genes in flowering using transcription inhibitors of protein [18] which is widely used in agricultural production. GA_3 in gibberellin was used in the experiment. The GA_3 concentration gradient was set as 10^{-3}M ~ 10^{-8}M. After repeated experiments, we found that the proportion of all flowers were at a low level, and the number of those flowering was basically within 3 (Figure 7-b). Thus, GA_3 is not suitable for *Wolffia australiana* floral induction.

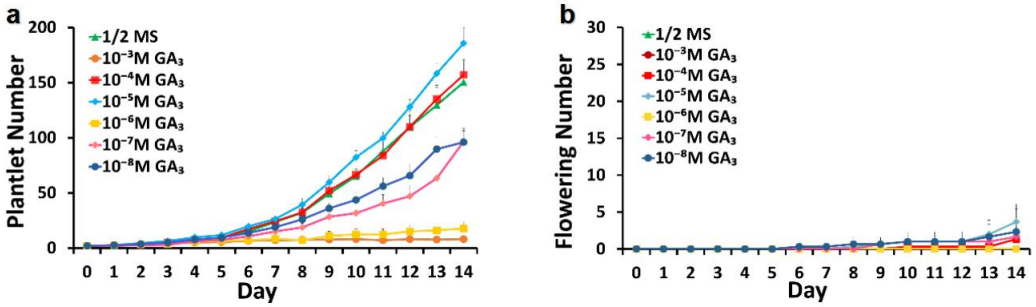

Figure 7 The plantlet number(a)、flowering number(b)of Wolffia australiana under GA_3 floral induction

2.3 The Flowering Number and Branching Number of *Wolffia australiana* Induced by a Combination of Reagents

Chemical reagents generally have complex interactions.To find a more efficient and stable treatment of floral induction, we combined these reagents to induce *Wolffia australiana* to flower.

2.3.1 The Flowering Number and Branching Number of *Wolffia australiana* Induced by the Combination of EDTA and SA

Based on the treatment results of the previous stage, the optimal concentration of EDTA and SA was selected as EDTA 10^{-4} M and SA 10^{-7} M,10^{-8} M,10^{-9} M for combined treatment. After 14 days, *Wolffia australiana* was in good growth condition and more flowering individuals were obtained (Figure 8-a,b).The experimental results showed that EDTA 10^{-4} M and SA 10^{-7} M were the best combination, and the number of flowering plants reached 25 (Figure 8-b).

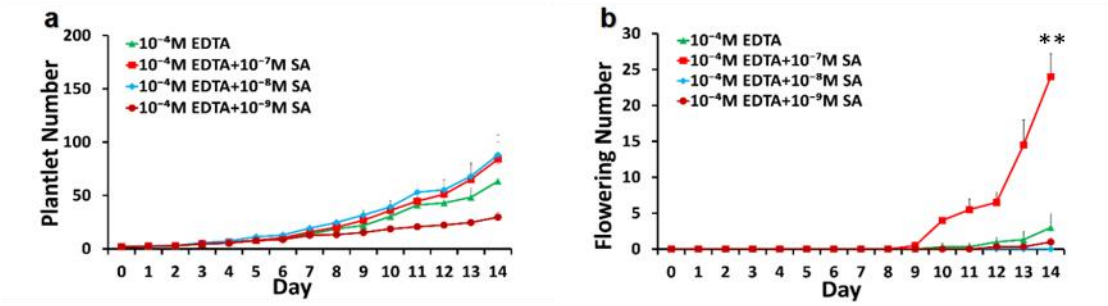

Figure8 The plantlet number(a)、flowering number(b)of Wolffia australiana under EDTA×SA floral induction

2.3.2 The Flowering Number and Branching Number of *Wolffia australiana* Induced by the Combination of EDTA and GA_3

The flowering number and branching number of *Wolffia australiana* treated with EDTA 10^{-4} M and GA_3 were mostly higher than those treated with EDTA 10^{-4} M alone (Figure 9-a, b).

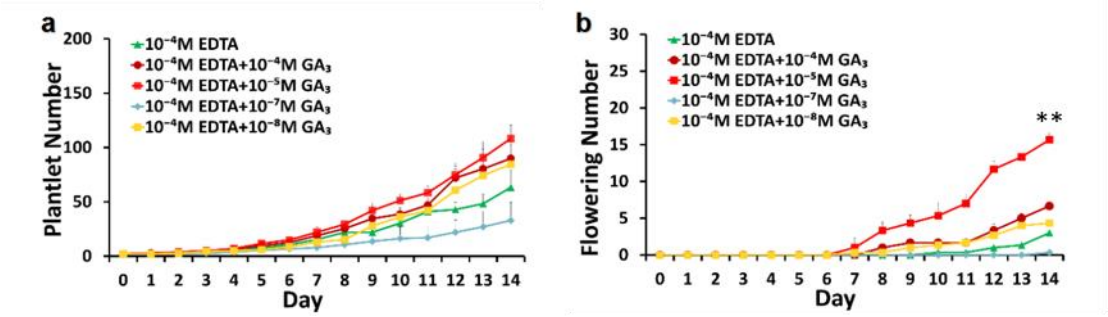

Figure9 The plantlet number(a)、flowering number(b)of Wolffia australiana under the induction of EDTA×GA_3

2.3.3 The Flowering Number and Branching Number of *Wolffia australiana* Induced by The Combination of GA_3 and SA

Having been treated with both SA and GA_3, *Wolffia australiana*'s growth was significantly inhibited as well as that of SA alone without flowering individuals (Figure 10). Therefore, we believe that the combination of SA and GA_3 has a poor effect on floral induction.

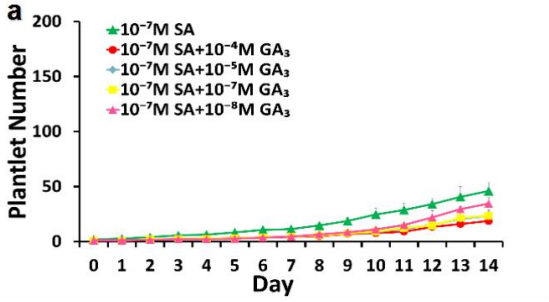

Figure10 The plantlet number(a)of Wolffia australiana under GA_3×SA floral induction

2.4 Establish the “Plant-on-Chip” Culture System Based on Microfluidics

The advantage of microfluidics is that it can accurately track the growth time and branching status of *Wolffia australiana* according to its position in the groove, and it has the characteristics of high efficiency and integration, so that multiple controlled experiments can be carried out simultaneously. Based on this, we designed the“Plant-on-Chip” culture system of *Wolffia australiana* (Figure 11), and first tracked the branching process of *Wolffia australiana* on the chip by time-lapse photography using a Stereo Microscope(Figure 12). According to the size of the individual *Wolffia australiana*, a groove slightly wider (about 0.9mm wide) than a *Wolffia australiana* plantlet, which was designed to ensure the growth of the *Wolffia australiana* along the trench branches. In addition, a “Plant-on-Chip” culture and recording system was constructed to track and monitor the growth status of the *Wolffia australiana* in real time (Figure 13)

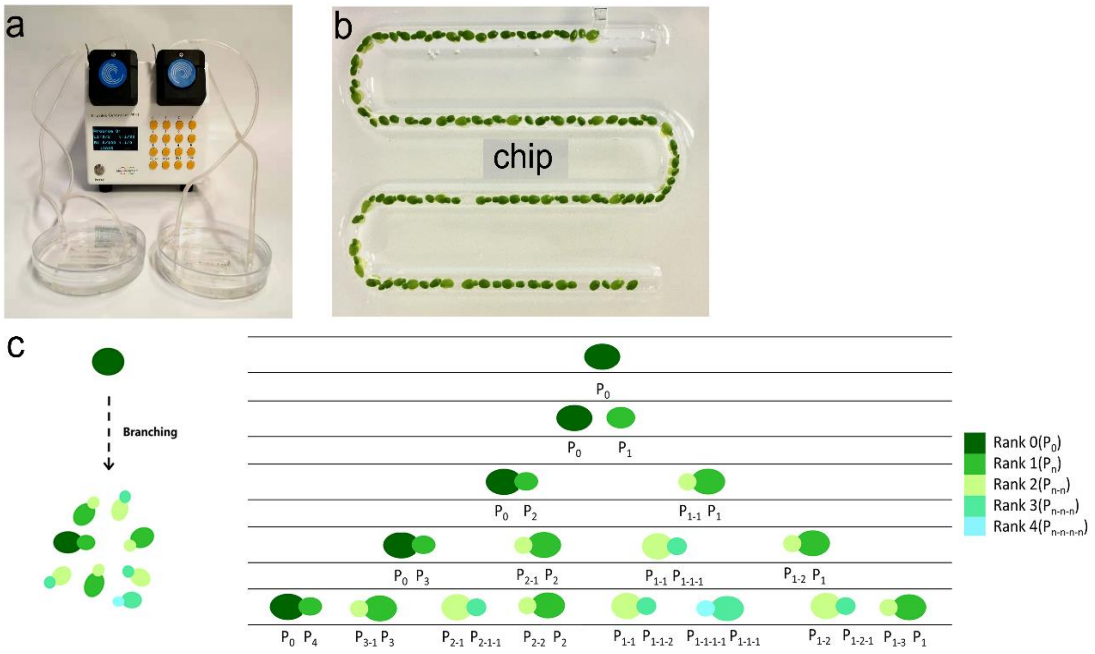

Figure 11 The physical diagram of “Plant-on-Chip”culture system and the branching diagram on the chip

(a)*The pump is connected to the petri dish through a hose. The pump rotates to circulate the liquid in the petri dish to avoid the accumulation of metabolic waste. The flow rate can be controlled by manually changing the parameters(b)Chips were placed in a petri dish, and Wolffia australiana branching sequentially on the chip. (c)A comparison of the Wolffia australiana branching patterns in the traditional petri dish and the“Plant-on-Chip”channel. Wolffia australiana of different colors represented individuals at different branching ranks, reflecting the locability of Wolffia australiana branch ranks in the “Plant-on-Chip”system.*

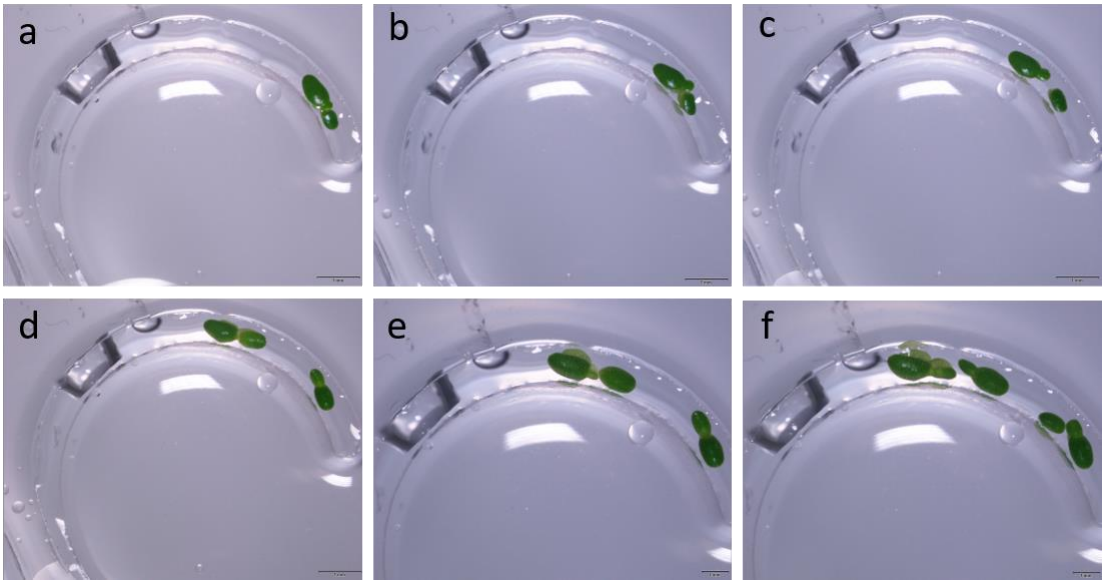

Figure 12 Reproductive growth process of Wolffia australiana under Stereo Microscope

a-f refers to the branching process of a single Wolffia australiana plantlet.The branch of the plantlet gradually moved to both ends, and the new one is constantly producing branches in the middle.

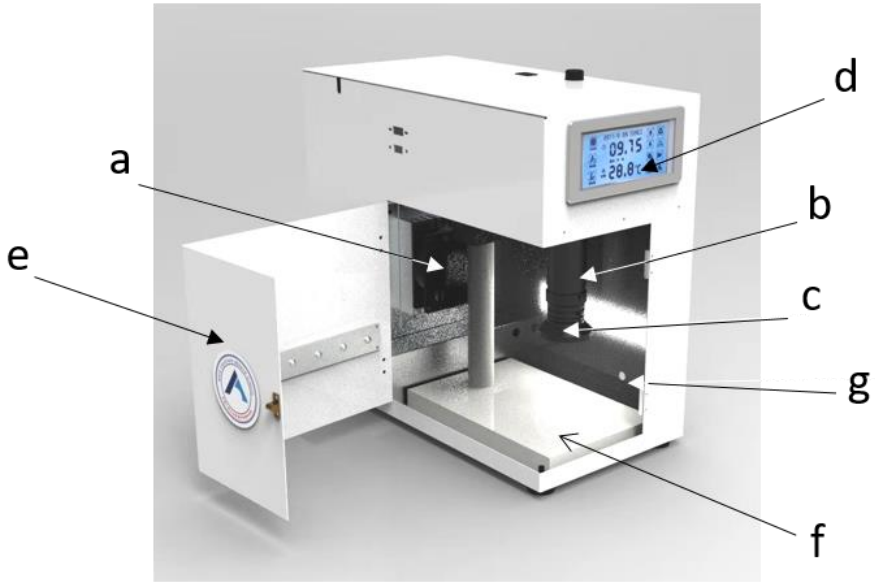

Figure 13 Incubator

(a) the built-in temperature control system (heater) and light control system (LED light strip) control the temperature and light intensity and time in the incubator. (b) (c) HD camera and electron microscope lens can collect dynamic video of chip and transmit it to computer via transmission line. (d) touch screen controller can set and change the cultivation conditions in the system in real time. (e) the hatch door allows easy placement and adjustment of petri dishes.(f)place to put petri dish(with chip and tubes).(g)the hole to allow tubes connect the microfluidic device

To explore the differences in *Wolffia australiana* gene expression at different stages

of EDTA floral induction, we designed a genome-sequencing experiment because the distance from the original *Wolffia australiana* represents the different stages of EDTA floral induction .

We sampled individuals from the 1/2MS Control group, the Flowering plantlet and the plantlet distance differently from the floral plantlet in EDTA induced group, setting 5 repetitions.With the help of RNA-seq technology, we detected the transitional differences in *Wolffia australiana* gene expression(Figure 14).

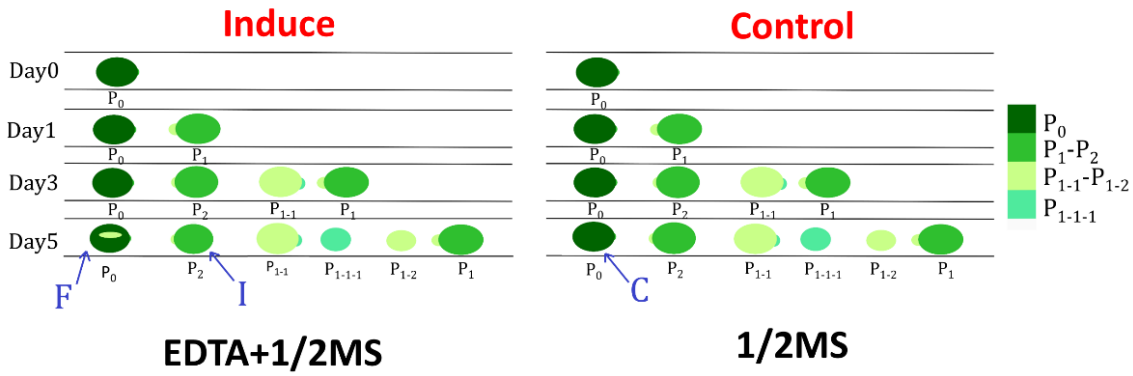

Figure 14 Schematic diagram of a small number of cells of a single Wolffia australiana sequence experiment design

The different colors represent the different branch ranks of Wolffia australiana, and one groove represents one branch.Among the sampled individuals indicated by arrows, C was the control group, F was the flowering individual treated by EDTA, I was the non-flowering individual induced by EDTA, and they were located at different distances from the flowering individual in the chip's groove.C, F and I individuals in each group were selected from five groups for repetition, and gene expression changes during induction of flowering were analyzed by detecting their gene expression changes.

2.5 Single *Wolffia austrialiana* RNA-seq and the Expression of Spectrum Analysis

After completion of *Wolffia austrialiana* flowering induction experiments on the chip, our cooperating partner Fuchou Tang's laboratory conducted the single *Wolffia austrialiana* RNA-seq experiment. Due to the strong technical advantage of Fuchou Tang's laboratory in the single-cell sequencing field, we obtained high-quality RNA and its library.All six of the biological duplicate samples have been sequenced(*Figure 15*).*Wolffia austrialiana* genome sequencing work is under way, more than 20000 genes have been annotated (cooperation laboratory unpublished data).Base on current progress, we have a great opportunity to uncover the basic flowering regulation network. The next step, according to RNA-seq results, will be to adjust our "Plant-on-Chip" culture system and optimize the sampling time point in the experiment, trying to find key regulatory factors in the early stages.

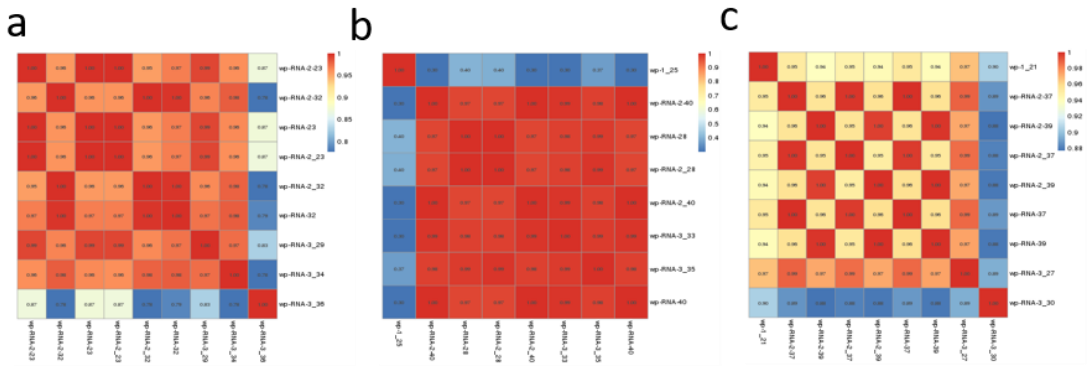

Figure 15 Quality analysis of the Single Wolffia austrialiana RNA-seq experiment quality analysis of reads in RNA-seq from Ling Li's laboratory, Mississippi university, USA. (a) correlation analysis between replicates in the Control group. (b) correlation analysis between replicates in the Flower group. (c) correlation analysis between replicates in the Induced group. The samples which have poor correlation(<90)with other replicates were discarded(Wp-rna-3_36 in the control group and Wp-1_25(F2) in the flower group.The correlation between wp-rna-3_30 and other replicates was slightly worse, but it was also up to 88%, which could be reserved for subsequent analysis.)

2.6 Determination of Element Content in *Wolffia australiana* before and after EDTA Treatment

EDTA treatment has a significant effect on *Wolffia australiana* induced flowering, but we have little understanding of the effect of EDTA on plant flowering. As a chelating agent, EDTA can chelate metal ions and lead to change in metal element content. To explore how EDTA induces *Wolffia australiana* flowering and whether it is related to the changes in the content of specific metal elements, we conducted a quantitative analysis of the changes in the content of metal elements in *Wolffia australiana* after EDTA treatment.

After EDTA treatment, the content of metal elements in *Wolffia australiana* was generally decreased. The content of Zn^{2+} was the most significant, turning to about 40% of the original (Figure 16). The decrease was also the largest (Figure 17), with a decrease of 171 microns /ml. The reduction of sodium and manganese was 20%. Magnesium reduced by about 10%; the proportion of boron increased by 15%, while the proportion of iron and calcium also increased to less than 50%. In addition, we did not detect Cu^{2+} ion in *Wolffia australiana.* This indicated that Satish C, Maheshwari and O. S. Chauhan's guesses on the mechanism of action of EDTA related to Cu^{2+} ions [14] were inconsistent with the experimental results.The experimental results of trace metal element content changes provide us with extremely strong evidence to ue to analyze the results of expression profile sequencing.

We will focus on the family of proteins that are closely related to these metals. For example, protein family genes with Zn^{2+} finger structure, Mn^{2+} and Mg^{2+} related genes involved in photosynthesis, and so on.

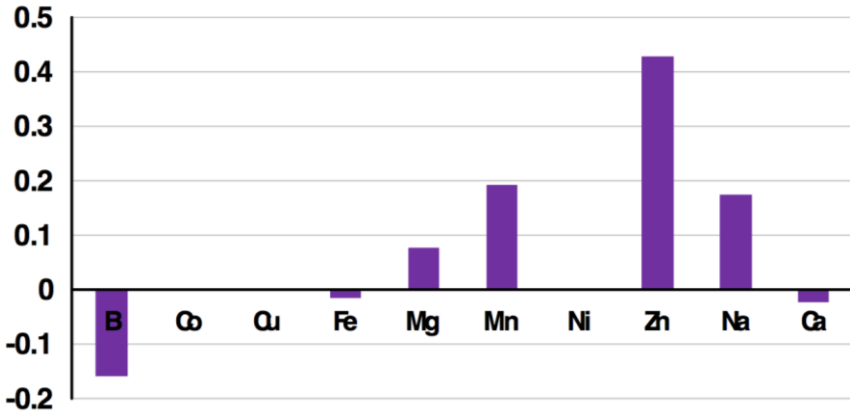

Figure 16 The Proportional Reduction of Metal Ions in Wolffia austrialiana after the treatment of EDTA

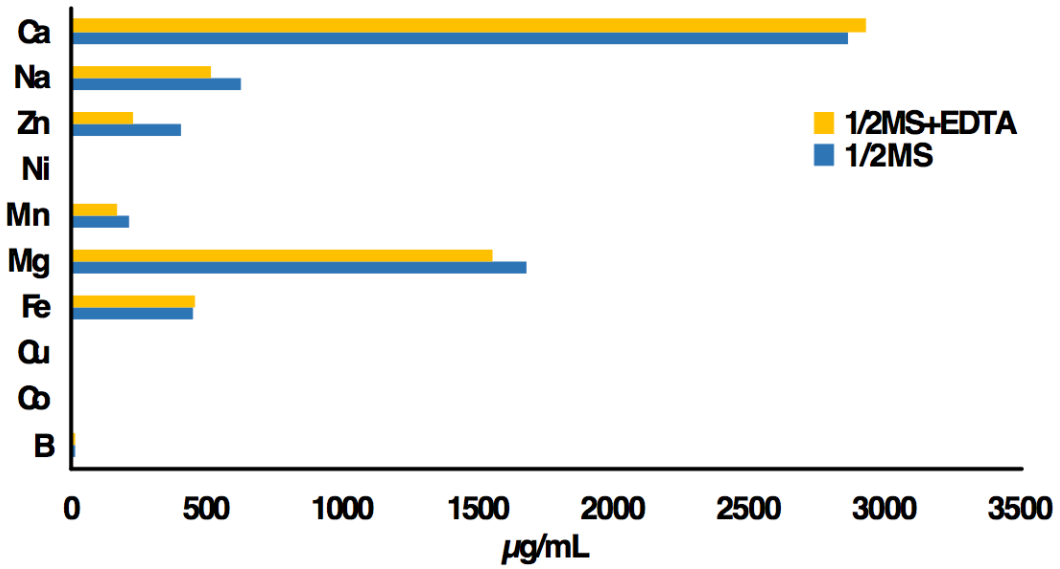

Figure 17 The Changes of Metal Ions in Wolffia austrialiana after the treatment of EDTA

3.Discussion

In order to establish *Wolffia australiana* as a new model plant, we conducted a comprehensive study on the life cycle of *Wolffia australiana* in the laboratory. First, we made a systematic morphological observation on *Wolffia australiana*, and recorded its branching, flowering and other life processes in detail. At the same time, we systematically induced *Wolffia australiana* to flower, and found out that 10^{-4} M EDTA and 10^{-7} M SA were the optimal treatments. These treatments allowed us to explore a stable and reliable floral inducing method for *Wolffia australiana*. In addition, the mechanism of action during inducing reagents EDTA was explored, and we discovered that a significant reduction of Zn^{2+} in *Wolffia australiana* might be the key factor of EDTA floral induction.

Moreover, in order to solve the problem that the traditional triangular bottle or petri dish culture system could neither locate nor track the individuals of *Wolffia australiana,* we 1) established a new "Plant-on-Chip" culture system by culturing these multicellular organisms on the chip for the first time, and 2) successfully set up the trinity culture system of positioning,cultivation and photographing, which lays a solid foundation for future research.

After that, we 3) combined the system with our previous physiological experiments to detect the gene expression differences of *Wolffia australiana* in different stages of reproductive growth by a small number of cells of a single *Wolffia australiana* sequence. This procedure laid a solid foundation for the subsequent analysis of gene regulation of flowering pathway. We thus completed the important step of whole *Wolffia australiana*'s life cycle under laboratory conditions.

Wolffia australiana is distinguished from other clonal plants by its unique branching pattern. The study of *Wolffia australiana* makes it possible to separate unitary plant development units for research. *Wolffia australiana* is not only a good subject for future research on plant development, but it also enabled us to have a deeper understanding of the nature of plant development. Under "Plant-on-Chip" culture system, we can also continue to explore how the apical meristem is formed, the reason why *Wolffia australiana* has no root, and how to form roots and,how plants respond to stress, etc. The application of microfluidic technology in the culture of *Wolffia australiana* holds great promise.

4.Experimental materials and methods

4.1*Wolffia australiana*,from the Institute of Hydrobiology, Chinese Academy of Sciences.Artificial incubator (Panasonic, mlr-352h-pc), temperature 26℃, 8h light /16h dark, light intensity 22000lux.

4.2Medium Arrangement

MS powder (sigma), sucrose (Beijing chemical plant).1/2MS 1% sucrose medium was prepared, pH meter (sartorius) was adjusted to about 7-8, and was sterilized at 121℃ for 15min in a high-temperature sterilizing pot (mls-3781l-pc).

4.3The Reagent

EDTA, SA, GA_3 treatment

Culture dish (90mm diameter) was added with 20ml MS medium, and a certain amount of 0.1%M or 0.04%M of reagent mother liquor was added respectively to form 10^{-3}M~ 10^{-9}M solution.After the reagents were fully mixed with the medium, one microping with the next branch was transferred from the MS culture environment, which was recorded as 2 on day 0.Three repeated experiments were performed for each concentration gradient.The number of individuals and the number of flowers were counted once a day for 14 days. Finally, the branch rate curve, the number of flowers and the proportion of flowers were made according to the average number of repeated groups in each gradient.Finally, the culture conditions suitable for *Wolffia australiana* to induce flowering were selected based on the growth state, branching rate and flowering ratio of *Wolffia australiana*.

4.4Video shooting

Wolffia australiana was photographed in focus under a stereoscopic lens (Olympus, sz2-ilst) on the microfluidic platform.Set the photo taking interval to 10min and the total duration to 72-168h.

4.5.Video List

Video 1 The growth process of *Wolffia australiana*.24h 7200X,scaleplate 1mm

Video 2 *Wolffia australiana* individual flowered on microfluidic platform.32h 7200X,scaleplate 1mm

4.6Material fixation and scanning electron microscope

(1)FAA fixing (100% alcohol: 50ml, 37-40% formaldehyde: 10ml, acetic acid: 5ml). Fixing steps include: Take material and soak the material in a fixative (aldehyde fixative); Use a vacuum pump to gently aspirate until the material sinks into the fixative; Fix for 12-24 hours, change fixative once during the process.

(2)Dehydration: the general procedure is as follows: 50% ethanol 20-30min; 70% ethanol 20-30min; 90% ethanol 20-30min; 100% ethanol twice, each time 20-30min.

(3)Critical point drying: the material was transferred to anhydrous copper sulfate - alcohol solution, into the sample basket. Alcohol immerses the material. during the

whole process, the material cannot dry.
(4)Spraying gold 90s: 15nm thickness (relatively thick, to avoid low or uneven conductance).
(5)Scanning electron microscope (Hitachi TM3030) photographing.

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