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Plasma-Activated Water Modulates Root Hair Cell Density via Root Developmental Genes in *Arabidopsis thaliana* L.

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Abstract: Low-temperature atmospheric pressure plasma technology has been used in agriculture and plant science by direct and indirect treatment of bio-samples. However, the cellular and molecular mechanisms affected by plasma-activated water (PAW) are largely unexplored. In this study, PAW generated from a surface dielectric barrier discharge (SDBD) device was used for plant development. Physicochemical analysis was performed to confirm the PAW properties that correlated with the plasma treatment time. *Arabidopsis thaliana* L. was utilized to study the effect of the PAW treatment in the early developmental stage. The plasma-activated water samples are denoted as PAW5 time in minutes (min), PAW7 min, PAW12 min, PAW19 min and PAW40 min with the plasma treatment time. Seedlings grown in the PAW5, PAW7 and PAW12 had increased root lengths while the root lengths were decreased in the PAW19 and PAW40. In the cellular level observation, the PAW treatment specifically increased the root hair numbers per unit of the root but suppressed the root hair length in the PAW, indicating that PAW mainly modulates the root hair cell density in the root. Furthermore, we found that the root hair density and length at PAW5 in maximal observed conditions were positively regulated by root developmental-related genes including *COBRA-LIKE9* (*COBL9*), *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE9* (*XTH9*), *XTH17*, *AUXIN1 (AUX1)* and *LIKE-AUXIN* (*LAX3)*.

Keywords: plasma agriculture; plasma-activated water; *Arabidopsis thaliana* L.; nitrate; root cell number

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1. Introduction

Low-temperature, atmospheric pressure, plasma technology has emerged as a new technology that potentially enhances plant growth and defense [1–3]. Bio-samples can be treated by a plasma device through gas or water [4]. Plasma-activated water (PAW) treatment changes the chemical properties, and PAW containing an altered chemical composition is utilized with plants [5,6]. In this indirect method, the development of low-temperature plasma technology is used for plant cultivation in agronomy [7–9]. PAW has differences in electrical conductivity, pH, nitrite, nitrate and H_2O_2 compared with deionized water (DW). As for the application of PAW in plants, ionized oxygen and nitrogen species dissolve in the water to create an acidic environment due to a lower pH [10,11]. Moreover, water treated with low-temperature plasma contains a high concentration of nitrate. Therefore, it has the role of a nitrogen (N) source for plant growth and could be possibly used as a liquid fertilizer [12,13]. Nitrogen is an important macronutrient

for plant growth and an essential component in the basic metabolic process [14–16]. The use of nitrogen fertilizer has a critical role in the green revolution by improving plant yield. Several reports have been published on using plasma treatment as an eco-friendly technology that produces nitrogen and functions as a liquid fertilizer [12,13,17,18].

Direct plasma application changes the appearance of the seed coat surface, resulting in a reduced time for germination and rapid growth in the development stage [7,19]. In tomatoes, a horticultural crop, stem growth differs from the controls according to the helium plasma treatment time [20]. The difference between the germination rate and growth rate for PAW in *Raphanus sativus, Solanum lycopersicum* and *Capsicum annum* has been reported to be due to the effects of a long PAW treatment time [18]. Both direct and indirect processing of plasma is considered to have a positive effect on plant development [21,22].

We selected *Arabidopsis thaliana* L. (*Arabidopsis*) as a model plant to understand the effect of PAW on its molecular mechanism during the plant development process. The growth cycle of *Arabidopsis thaliana* L. is very short, 4–6 weeks. In addition, the number of seeds in the plant is about 1500, which is a good model for studying genetics. *Arabidopsis* has a relatively small 135 Mbp genome, 5 pairs of chromosomes and 33,602 genes, which make it easy to analyze the genome, and can be used for other crop applications. The *Arabidopsis* genome sequence was first decoded in December 2000. Since then, the advancement of plant science has accelerated at a faster pace [23,24]. This advancement was due to the influence of *Arabidopsis* as a good model plant to understand the fundamental phenomena of plants.

In this study, we examined the phenotype, cytological observation, and gene expression after PAW treatment to elucidate the molecular mechanism of *Arabidopsis* during the development process. As a plasma generating device, the plasma-activated water was generated using a surface dielectric barrier discharge (SDBD) to dissolve ionized gas in deionized water. In particular, hydrogen peroxide (H2O2), nitrate (NO3-), pH and electrical conductivity were altered during the plasma treatment time. The altered physicochemical properties affected the vegetative and root development. In the cotyledon, PAW treated palisade cells were regulated by cell expansion, not cell proliferation, and the root hair number was dramatically increased in the root cells. In the present study, we deduced that PAW modulates the root hair cell density induced by the expression of root developmental-related genes, COBL9, XTH9, XTH17, AUX1 and LAX3.

2. Materials and Methods

2.1. Plasma-Activated Water Preparation and Physicochemical Analysis

The plasma source used in this study was previously described in Lee et al., 2020 [25]. A surface dielectric barrier discharge (SDBD) in an air-tight container was used as the plasma treatment device. The electrodes of the SDBD generator were made up of two parallel metals, one of which was covered with a dielectric layer, and the plasma was generated using an alternating current (AC) flowing through the electrodes. The electrode consisted of powered and grounded stainless steel, and an aluminum oxide plate (1 mm-thick) was placed between the electrodes. Each electrode was attached at the top of the reactor with a 10 W average consumed power, an 8 kVpp voltage amplitude and a 17 kHz frequency. A 12 cm fan below the electrodes was used (15-LED 120, Aone, China) to circulate the air inside the reactor. In the reactor, the distance between the two electrodes and the water surface was 12 cm. The optical emission spectrum (OES) was obtained by an ultraviolet–visible (UV-VIS) spectrometer (Oceanoptics, maya 2000 pro) within the range of 200–600 nm. An optical lens (Ocean optics, UV-74) was used to collimate the emission spectrum. The lens was placed in front of the SDBD at 1.5–2.0 cm. The OES was obtained with an integration time of 1 s which was averaged 50 times.

PAW was produced by adding 1 L of deionized to the reactor. The PAW was prepared according to the plasma treatment time for 5, 7, 12, 19 and 40 min The plasma-activated water samples in this paper are denoted as PAW5, PAW7, PAW12, PAW19 and PAW40 with the number indicating the plasma treatment time.

To confirm the chemical properties of the PAW, the anion content was measured one day after PAW generation using ion chromatography (ICS-2100, Thermo Dionex, Sunnybale, CA, USA). To quantify the anions, the standard sample [Dionex Seven Anion Standard II (in deionized water)] was measured with a three-point method (25, 50, and 100 mg/L) [24], and the pH and conductivity were measured using Orion™ Versa Star Pro™ (Thermo Scientific, Waltham, MA, USA). To meet the appropriate pH for plant growth, the pH was adjusted to pH 5.6~5.8 with 0.1 N and 1 N KOH solution. An AQUAfast AQ3700 Colorimeter (ORION AQ3700, Thermo Scientific Orion, USA) was used to measure the hydrogen peroxide (H_2O_2) using the hydrogen peroxide LR method (Tintometer Lovibond, UK). All chemical properties measurement was performed using at least 3 replicates.

2.2. Plant Material and Growth Conditions

To prevent contamination, the *Arabidopsis thaliana* L. (Columbia 0, Col-0, ecotype background) seeds were surface sterilized with 20% commercial bleach followed by washing with sterilized deionized water and then stored at 4 °C for 3 days for the cold treatment before placing them onto the media. The sterilized seeds were placed onto agar medium and allowed to dry for 30 min before moving the plates into the growth chamber. The growth chamber setting was set at 22 °C, 60% relative humidity, and 16 h/8 h, light/dark condition, respectively. The seeds were in the growth chamber for 15 days. Solid medium was prepared using plant agar (Duchefa Biochemie) at a 0.8% concentration with deionized water (DW) and PAW (5, 7, 12, 19, 40 min). Seedlings were grown on the minimal growth condition media at the vertical position for the root length examination and the horizontal position for the cotyledon size examination. All sterilization and planting procedures were carried out on a clean bench. To observe the root morphology, the midpoint in the longitudinal direction of the root was observed for the optimal root hair initiation and length phenotype.

WER::GFP/cobl9-1 (CS67758) and *cobl9-1* (Salk_099933) were acquired from the *Arabidopsis* Biological Resource Center (ABRC), www.arabidopsis.org (accessed on 2 March 2021), to examine the root hair. Two T-DNA insertional mutants, *WER::GFP* and *cobl9-1*, were Col-0 background. The double mutant *WER::GFP/cobl9-1* (CS67758) was obtained by crossing the single loss of mutant line (*cobl9-1*, Salk-099933) and the *WER::GFP* mutant. The *COBRA-LIKE 9* (AT5G49270) gene is involved in root hair [26,27]. Genotyping was performed using polymerase chain reaction (PCR) to confirm the deletion of the *cobl9-1* mutant and the insertion of the *GFP* gene. AccuPower Taq PCR PreMix (BIONEER) was used, and the temperature and time were as follows: 95 °C for 5 min for the initial denaturation, 35 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min, and a final extension step of 72 °C for 5 min Gel loading was performed using 1xTAE, Red safe (1×), and 1.5% Agarose (Duchefa Biochemie, The Nederland).

2.3. Morphological Analysis

To count the palisade cells and measure their size in the cotyledon, samples were prepared from 10-day-old *Arabidopsis* grown in the horizontal growth condition. The cotyledon leaves were preserved in FAA solution (5% acetic acid, 45% ethanol and 5% formaldehyde) and fixed at 4 °C overnight. An ethanol series (50%, 60%, 70%, 80%, 90%, 95%, 99% and 100%) was sequentially used for the sufficient dehydration. Then, a 50:50 ratio of ethanol and methyl salicylate (Sigma-Aldrich, St. Louis, MI, USA) was added to the leaves which then sat for an hour, and finally, 100% methyl salicylate was added and substituted. The prepared cotyledon leaves were fixed using slide glass and cover glass, and the palisade cells of the cotyledon were photographed using a differential interference contrast (DIC) microscopy (ZEISS) system to count the number and measure the size.

2.4. Microscopy

To confirm the appearance and shape of the root hair development of *Arabidopsis thaliana* L., Columbia 0 (Col-0) and *WEREWOLF* (*WER)::GFP/cobl9-1* (CS67758) were grown in Petri

dishes for 5 and 7 days. The root morphology was examined using a Nikon-Fi3 stereo microscope with a 5× zoom magnification, objective lens (10×), 40 ms exposure, and 2.0× analog gain. To check the GFP fluorescence of the *WER::GFP/cobl9-1* lines, we selected the middle area of the roots with the optimal root hair initiation and elongation present. The GFP signals were detected under a Nikon-A1 confocal microscope at 488 nm with eyepiece lens (10×), objective lens (20×) and a FITC filter with the laser set to 40% and the analog gain to 80%. The quantification was performed using the photograph image of the microscopy.

2.5. RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Analysis

For the gene expression analysis, the samples were grown for 15 days and then sampled and immediately frozen in liquid nitrogen and crushed using a bead beater followed by RNA extraction from the roots using the Trizol reagent (Thermo Fisher Scientific, USA) method. The purified RNA was then treated with the Turbo DNA-free kit (Invitrogen, Carlsbad, CA, USA). For the reverse transcription, 500 ng of RNA were used for the cDNA synthesis using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen, USA) following the manufacturer's instructions. Then, 1 µL of cDNA was used for real-time PCR, and the results were quantified with the CFX Manager™ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA, http://www.bio-rad.com/ (accessed on 2 March 2021)), and Ubiquitin 10 was used for normalization. The primers used in this study are listed in Supplementary Table S1.

3. Results

3.1. Plasma Diagnostics and Plasma-Activated Water (PAW) Characteristics

The emission spectrum of the plasma from SDBD was measured using optical emission spectroscopy (OES). The result shows the intensive molecular spectra of a nitrogen second positive system (N_2 SPS) and nitrogen first negative (N_2 FNS) in the 300–400 nm region during the air discharge. An OH radical peak (309 nm) was not observed; thus, the air discharge may not produce OH radicals (Figure 1). Because N_2 SPS and N_2 FNS were detected by OES, it is estimated that a large amount of nitrogen ions will be generated by this plasma system. When these NO_x react with H_2O, they become ionized into $NO2^-$ and $NO3^-$, which were detected in the PAW. The physical and chemical properties of DW and the PAW were analyzed (see the Materials and Methods section), and the results are presented in Table 1. To produce the proper PAW for *Arabidopsis*, plasma-activated water samples were generated using five different PAW treatment times and used in the experiment. The plasma-activated water samples in this paper are denoted as PAW5 time in minutes (min), PAW7, PAW12, PAW19 and PAW40 with the number indicating the plasma treatment time.

The result showed that the pH decreased (increased acidity) as the PAW processing time was extended due to the change in the ion content of the water. Furthermore, we confirmed that a small amount of nitrite was detected in the PAW, whereas the concentration of nitrate ions and their conductivity dramatically increased in proportion to the plasma treatment time in the PAW. In the case of hydrogen peroxide (H_2O_2), it was not detected in the DW, but it increased slightly depending on the plasma treatment time (Table 1).

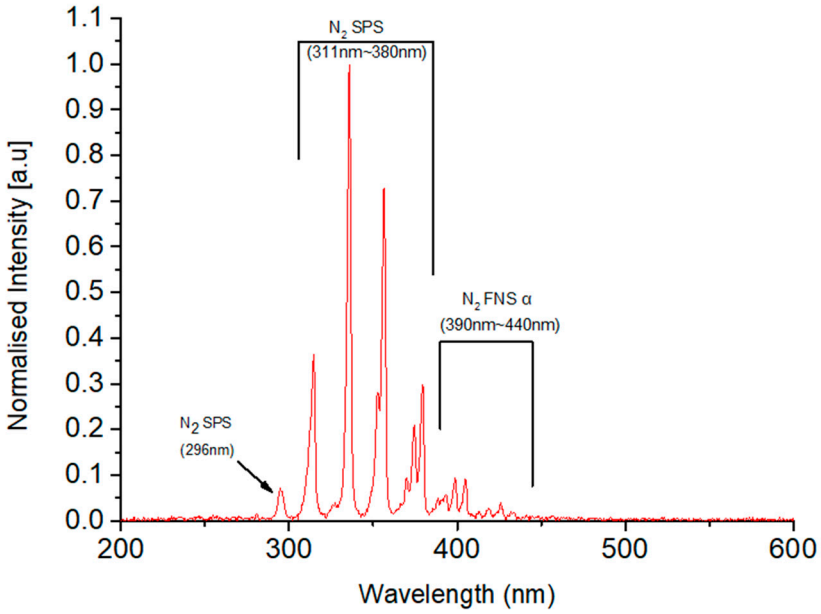


Figure 1. Optical emission spectrum of the surface dielectric barrier discharge (SDBD) with air from 200 to 600 nm.

Table 1. Physicochemical properties of the plasma-activated water (PAW).

	pH	Nitrite NO_2^- (mg/L)	H_2O_2 (mg/L)	Nitrate NO_3^- (mg/L)	Conductivity (μs/cm)
Deionized Water(DW)	5.74 ± 0.06	0.00	0.00	0.00	1.53 ± 0.13
PAW5 min(PAW5)	3.62 ± 0.02	1.09 ± 0.11	0.09 ± 0.01	25.29 ± 2.88	118.10 ± 2.26
PAW7 min(PAW7)	3.34 ± 0.03	1.24 ± 0.12	0.14 ± 0.01	49.05 ± 2.61	218.50 ± 9.64
PAW12 min(PAW12)	2.94 ± 0.08	1.85 ± 0.07	0.27 ± 0.02	102.67 ± 6.30	460.33 ± 15.25
PAW19 min(PAW19)	2.62 ± 0.07	3.68 ± 0.12	0.88 ± 0.04	204.87 ± 8.74	972.93 ± 32.41
PAW40 min(PAW40)	2.37 ± 0.04	5.17 ± 0.16	1.31 ± 0.04	389.08 ± 12.24	1847.00 ± 70.19

The results of the Table 1 are the mean ± standard deviation (SD).

3.2. PAW Increases the Total Root Length and Root Hair Number in the Arabidopsis Root

To understand how the PAW treatment affects the kinetics on root elongation in *Arabidopsis* during development, we monitored the changes in root length using solid media to see a more severe and significant effect of PAW component affecting the growth. The changes in the compositions of the nitrogen ions in the PAW were predicted to affect plant growth and development. As the result shows, the root length became significantly longer with the PAW5, 7, and 12 treatments from day 7 to day 15 after planting compared to the DW treatment (Figure 2). In contrast to the root length for the PAW5, 7, and 12 treatments, the root length was significantly shorter for the PAW 19 and 40 treatments compared to the DW treatment. At 7 days after planting, the root lengths in the PAW5 treated plants were increased to about 33% compared to DW (Figure 2). Furthermore, at 15 days after planting, the root lengths for the DW-treated seeds were 22.14 ± 2.62 mm, and for PAW5, they were 41.38 ± 3.44 mm showing an 87% difference in length (Figure 3). In contrast, the root lengths under the PAW19 and 40 conditions were suppressed compared to the DW treatment (Figure 2). The root length at 7 days after planting was 9.82 ± 1.07 mm, which was about 28% shorter compared to the DW treatment, while at 15 days after planting, it was 39% shorter, indicating that the PAW40 condition was the most suppressed condition. It is indicated that the appropriate plasma treatment time is essential for plant growth and development, and if excessive, it inhibits growth (Figures 2 and 3).

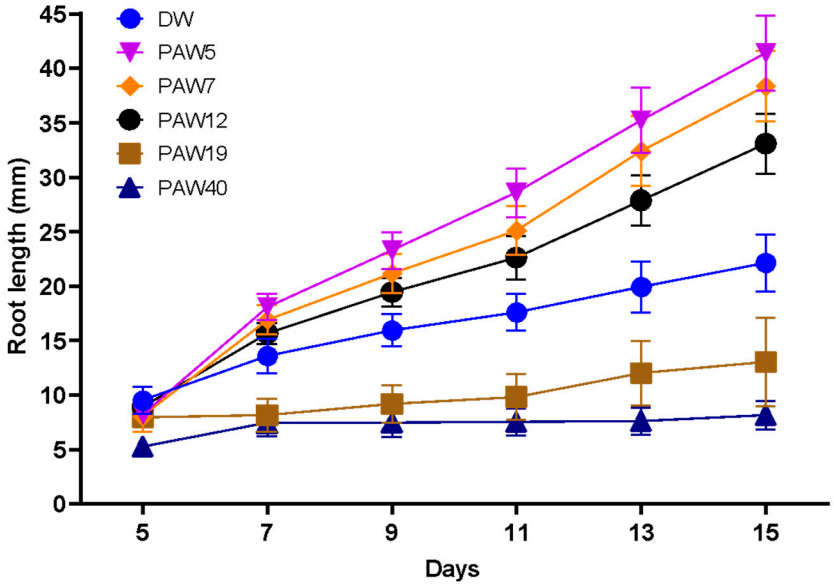


Figure 2. Plasma-activated water promotes root elongation during early root development in *Arabidopsis thaliana* L.

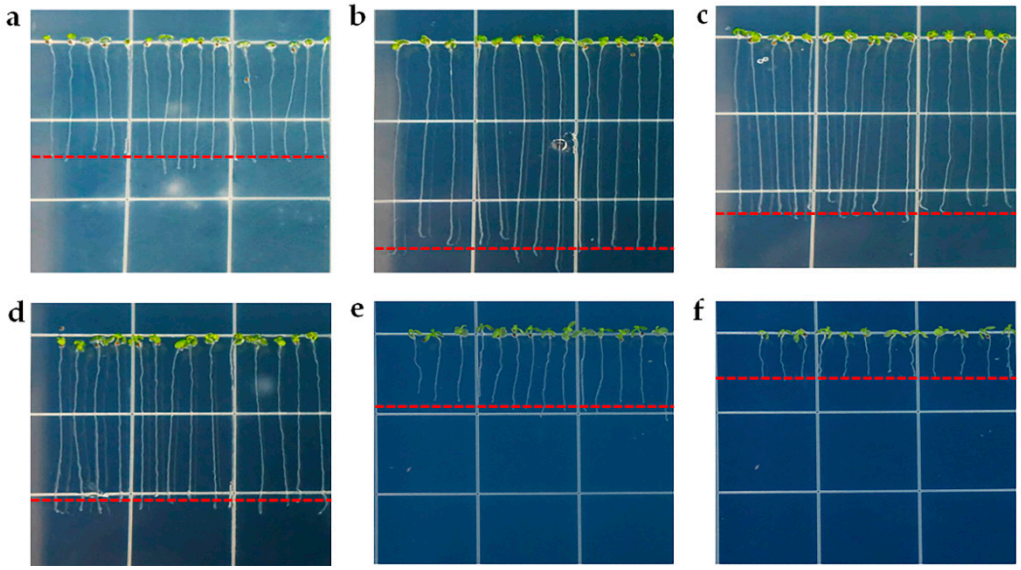


Figure 3. PAW effects on root length during the developmental stage of *Arabidopsis thaliana* L. Seedlings were vertically grown for 15 days on agar medium. (**a**) DW, (**b**) PAW5, (**c**) PAW7, (**d**) PAW12. (**e**) PAW19, (**f**) PAW40. One square size indicates 2 × 2 cm.

Because the difference in the root length was only clearly visible 7 days after planting, we further checked more detailed morphological characteristics in the earlier stage of plant growth. At 5 days after planting, Col-0 grown on the plant medium did not show any differences in the root length. However, under closer examination using a stereo microscope, the number of root hairs increased by 84.83 ± 9.21 for the DW treatment and by 112.00 ± 5.25, 107.83 ± 5.74, 100.50 ± 8.11 and 92.66 ± 6.18 for the PAW5, PAW7, PAW12, and PAW19 treatments, respectively (Table 2). Furthermore, the length of the root hairs was 550.01 ± 138.61 µm for the DW treatment and 284.06 ± 63.12, 188.12 ± 46.67, and 161.50 ± 38.05 µm for the PAW5, PAW12, and PAW19 treatments, which are shorter than that of the DW treatment by about 48% and 71% for the PAW5 and PAW19 treatment, respectively, (Table 2, Supplementary Figure S1,). Therefore, the results show that the PAW

treatment increases the number of root hairs while it shortens the length of the root hairs at 5 days after planting.

Table 2. Total root length, root hair number and root hair length *A. thaliana* L. (Col.0) 5 days after planting.

	DW	PAW5	PAW7	PAW12	PAW19
Root length (mm)	9.91 ± 0.85 (n = 15)	8.19 ± 0.68 ns (n = 15)	8.04 ± 1.40 ns (n = 15)	8.92 ± 0.82 ns (n = 15)	7.95 ± 0.77 ns (n = 15)
Root hair number	84.83 ± 9.21 (n = 6)	112.00 ± 5.25 *** (n = 6)	107.83 ± 5.74 *** (n = 6)	100.50 ± 8.11 ** (n = 6)	92.66 ± 6.18 ns (n = 6)
Root hair length (μm)	550.01 ± 138.61 (N = 100)	284.06 ± 63.12 *** (N = 100)	193.20 ± 33.45 *** (N = 100)	188.12 ± 46.67 *** (N = 100)	161.50 ± 38.05 *** (N = 100)

The midpoint in the longitudinal direction of the root was observed for the root hair number and root hair length by examining the most optimum root hair initiation and length phenotype. For the root length and root hair number, "n" indicates the number of individual plants. For root hair length, "N" indicates the individual root hair measured from total 6 plants. The results of the Table 2 are the mean ± SD (Student's *t*-test compared with D.W. $p > 0.05$ ns, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

3.3. PAW Treatment Increases the Cotyledon Size Dependent on Palisade Cell Enlargement

To further confirm the PAW effect on early vegetative development in *Arabidopsis*, the cotyledon was selected for cellular level examination. The size of the cotyledon was significantly increased in the PAW treated samples as examined by stereo microscope (Figure 4a–d). Quantification of the cotyledon size confirmed the enlargement of the cotyledon in the leaf length and leaf width directions for all PAW treatment conditions (Table 3). It was found that the cotyledon size for the PAW40 treatment condition was increased by about 60% in the leaf length direction (longitudinal direction) and by about 65% in the leaf width direction compared to the DW treatment condition.

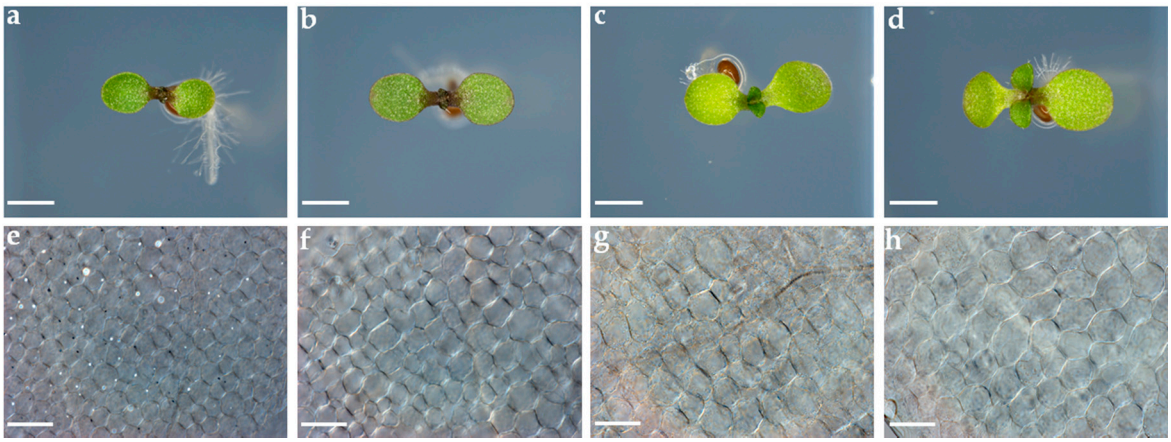


Figure 4. PAW treatment increases the *Arabidopsis* cotyledon dependent on the palisade cell size. (**a**–**d**) cotyledon size examined by a stereo microscope. (**e**,**f**) Palisade cells were examined from a cleared cotyledon sample under a differential interference contrast (DIC) microscope. (**a**,**e**) Deionized water (DW), (**b**,**f**) PAW5, (**c**,**g**) PAW19, (**d**,**h**) PAW40. (**a**–**d**) Bar = 1000 μm. (**e**–**h**) Bar = 50 μm).

There are two important factors that generally determine plant morphology. One is the number of cells involved in cell proliferation, and the other is the size of the cells involved in elongation [28]. To determine the main factor that affects the size of the cotyledon, we observed the cell size and number of the palisade cells of the cotyledon using the clearing method and examined it under a DIC microscope (Figure 4e,f). As shown in Table 3, the difference in the number of palisade cells in the leaf length direction was not significant between the DW and PAW treatment conditions. However, the size of the palisade cells in the leaf length direction growth was 15.47 ± 1.50 μm in the DW treatment condition and 42.88 ± 3.36 μm in the PAW40 treatment condition, showing a 277% difference between DW and PAW40. The size of the palisade cells increased in proportion to the treatment time of

the PAW. Therefore, it was found that plasma-activated water modulates the morphology of the cotyledon, which is dependent on cell size regulation, not cell proliferation.

Table 3. Cotyledon size and palisade cell size and number of *Arabidopsis* grown in the DW and PAW treatment condition at 10 days after planting.

	DW	PAW5	PAW7	PAW12	PAW19	PAW40
Cotyledon size in the leaf length direction (mm)	0.60 ± 0.03 (n = 30)	0.77 ± 0.09 *** (n = 21)	0.81 ± 0.09 *** (n = 25)	0.84 ± 0.09 *** (n = 30)	0.88 ± 0.10 *** (n = 15)	0.96 ± 0.14 *** (n = 23)
Cotyledon size in the leaf width direction (mm)	0.46 ± 0.03 (n = 30)	0.62 ± 0.07 *** (n = 21)	0.64 ± 0.06 ** (n = 25)	0.67 ± 0.07 *** (n = 30)	0.63 ± 0.05 *** (n = 25)	0.76 ± 0.08 *** (n = 23)
Palisade cell number	48.20 ± 6.83 (n = 5)	43.20 ± 8.19 ns (n = 5)	45.40 ± 5.59 ns (n = 5)	47.40 ± 5.85 ns (n = 5)	48.40 ± 5.59 ns (n = 5)	40.40 ± 5.31 ns (n = 5)
Palisade cell size (μm)	15.47 ± 1.50 (N = 300)	24.63 ± 2.34 *** (N = 300)	25.07 ± 2.39 ** (N = 300)	25.59 ± 2.00 *** (N = 300)	31.35 ± 3.93 *** (N = 300)	42.88 ± 3.36 *** (N = 300)

For the cotyledon size and palisade cell number, "n" indicates the number of individual plants, and for the palisade cell size, "N" indicates the individual palisade cells measured from a total of 5 individual samples. Single cotyledons were measured from each biological sample. The palisade cell number was counted from the cotyledon length direction. Results are the mean ± SD (Student's *t*-test compared with DW; $p > 0.05$ ns, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

3.4. PAW Modulates the Root Hair Density through Root Developmental Genes

Root hair is one of the important organs in plants. It controls osmotic pressure to absorb moisture and increases the area where the root comes into contact with the soil [29]. In *Arabidopsis*, the development of root hairs is determined by the location of the epidermal cells [30]. The epidermis cells adjacent to two cortical cells develop into root hairs, while epidermis adjacent to one cortical cell does not develop into root hairs [31,32]. *GFP* fusions with target genes such as *GLABRA2 (GL2)*, *WEREWOLF (WER)*, and *CAPRICE (CPC)* expressed in the root epidermal have been used to study root epidermal patterns in a previous study [26]. The visualization of the GFP helps to differentiate the cell structure and development process and clearly shows any alteration in the root. In *Arabidopsis*, *cobl9-1* was shown to have root hair defects. The *COBL9* gene is one of the important biomarkers as a root hair-specific gene and has a crucial role in root hair formation during development [33]. By using the double mutant *WER::GFP/cobl9-1*, we expected to see a clear effect of the PAW treatment in epidermal cell patterning which can help us to understand the morphological structure at the single cell level, especially the root hairs. Furthermore, PAW treatment via *cobl9* can help to confirm if the root hair phenotype is dependent on a single pathway or broader gene regulatory networks.

The difference in the morphology of the root hairs between the DW and PAW treatments at days 5 and 7 of the developmental stage was confirmed using the *WER::GFP/cobl9-1* mutant line. The results showed at 5 days after planting that there were no differences in the root length, but the root hair number and root hair length were changed between the DW and PAW treatment. In detail, the number of root hairs was increased by 14.5 ± 1.37 in the DW treatment. This was increased by 40.66 ± 5.71 and 27.50 ± 5.54 for the PAW5 and PAW19 treatments, respectively. The length of the root hair was decreased by 138.35 ± 11.11 and 85.05 ± 11.65 ụm for the PAW5 and PAW19 treatments compared with 261.21 ± 100.37 ụm for the DW treatment. These results show that the PAW treatment did not affect the total root length, but the root hair phenotypes were altered for the plant harboring the mutation in the *COBRA-LIKE9* genes, especially during the early developmental stage. In addition, the length of the root hairs was shortened, but the number of root hairs was increased for all PAW treatment conditions (Table 4, Supplementary Figure S2).

Table 4. Root hair number and length of *Arabidopsis* (*WER::GFP/cobl9-1*) at 5 days after planting.

	DW	PAW5	PAW7	PAW12	PAW19
Root length (mm)	9.75 ± 0.96 (n = 15)	10.07 ± 0.75 ^{ns} (n = 15)	10.10 ± 0.63 ^{ns} (n = 15)	9.78 ± 0.95 ^{ns} (n = 15)	10.08 ± 0.66 ^{ns} (n = 15)
Root hair number	14.50 ± 1.37 (n = 6)	40.66 ± 5.71 *** (n = 6)	33.16 ± 3.6 *** (n = 6)	31.00 ± 3.84 ** (n = 6)	27.50 ± 5.54 ^{ns} (n = 6)
Root hair length (μm)	261.21 ± 100.37 (N = 60)	138.35 ± 11.11 *** (N = 60)	108.25 ± 11.54 *** (N = 60)	99.50 ± 13.65 *** (N = 60)	85.05 ± 11.65 *** (N = 60)

For root length and root hair number, "n" indicates the number of individual plants as biological replicates measured; for root hair length, "N" indicates the individual root hair length measured from a total of 6 samples. The results are the mean ± SD (Student's *t*-test compared with D.W. $p > 0.05$ ns, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

Because the differences in the root hair phenotype between the DW and PAW treatment conditions were significant at 5 days after planting (Table 4), a confocal microscope was used for imaging the root epidermal cell shape at 7 days after planting. To visualize the cell type in the root, we used three different treatment conditions, DW, PAW5, and PAW19. The roots of the *Arabidopsis WER::GFP/cobl9-1* line were grown for 7 days. Figure 5 shows the tagged *WER* promoter with *GFP*, which resulted in the appearance of fluorescence in all the epidermal layers of the root cells. Quantification of the root hair numbers showed an increment in all the PAW treatment conditions compared to the DW treatment condition. In contrast, the root hair length was decreased in the PAW treatment condition compared to the DW treatment condition (Table 5, Supplementary Figure S2). Interestingly, the length of the root epidermal cells was elongated with a similar pattern to the root length phenotype after the PAW treatment, which is consistent with the root enhancement in the PAW5 and PAW7 and the reduction in the PAW19 treatment condition compared to the DW treatment condition. It is believed that there will be a specific mechanism regulating the number of root hairs and length of the root hair in the PAW condition during the plant development process. Moreover, the results indicate that PAW5 is the optimized condition for plant growth and development.

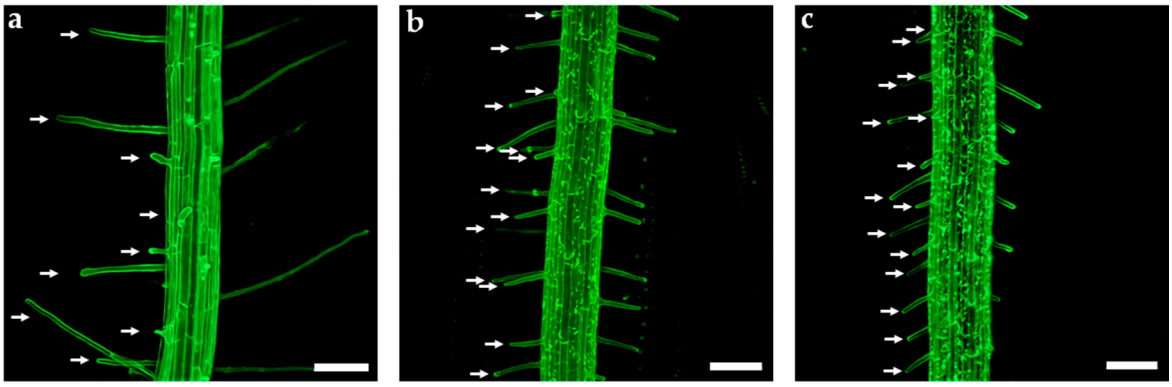


Figure 5. PAW regulates the number of root hairs and epidermal cell size in the *Arabidopsis* root (*WER::GFP/cobl9-1*). *Arabidopsis* seedlings were grown in solid media for 7 days. (**a**) DW, (**b**) PAW5, (**c**) PAW19. The white arrows indicate the individual root hairs that were used in the quantification. Bar = 100 μm.

To further understand the mechanism of PAW affecting the root development in *Arabidopsis*, we confirmed the expression of several marker genes related to root hair initiation and elongation. Previously, it was shown that the nitrate concentration could induce the expression of *COBL9* which activates root hair initiation and elongation [34]. *NT_COB9* was upregulated by PAW treatment in tobacco [25]. As expected, the expression of *COBL9* was significantly increased in the PAW5 treatment condition compared to the DW treatment condition. In contrast, the expression was suppressed in the PAW19 treatment condition. *AUXIN1 (AUX1)* and *LIKE-AUXIN* (*LAX3)* are the key auxin influx carriers and are multi-membrane transmembrane proteins that transfer the auxin involved in

cell growth [35]. *AUX1* and *LAX3* both had up-regulated expression patterns in PAW5 while *AUX1* was down-regulated in the PAW19 treatment condition. For *OBF BINDING PROTEIN 4* (*OBP4*), its expression was down-regulated in the PAW5 and PAW19 treatment conditions. *OBP4* is known as a negative regulator of root growth and differentiation including the root hairs [33,34], and it also functions as a negative regulator in PAW. Furthermore, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* (*XTH*) gene family members are factors involved in cell wall modification [36–38]. Interestingly, two genes, *XTH9* and *XTH17*, showed high expression for PAW5 but low expression for the PAW19 treatment condition (Figure 6). We concluded that the root architecture under PAW is modulated by root developmental genes including *COBL9*, *XTH9* and *XTH17*.

Table 5. Root hair number and length of *A. thaliana* L. *(WER::GFP/cobl9-1)* grown for 7 days after planting.

	DW	PAW5	PAW19
Root hair number	15.14 ± 2.47 (n = 7)	24.57 ± 3.10 *** (n = 7)	26.85 ± 5.33 *** (n = 7)
Root hair length (μm)	162.92 ± 29.10 (N = 50)	77.40 ± 7.21 *** (N = 50)	54.33 ± 7.83 *** (N = 50)
Root epidermal cell (μm)	133.01 ± 12.67 (N = 50)	141.33 ± 11.45 *** (N = 50)	110.46 ± 9.38 *** (N = 50)

For the root hair number, "n" indicates the number of individual plants; for the root hair length from the root epidermal cells, "N" indicates the individual root hairs measured from a total of 7 samples. The GFP signals for root hair umber, root hair length and root epidermal cell were measured under a Nikon-A1 confocal microscope at 488 nm with a eyepiece lens (10×), objective lens (20×). The results in the Table are the mean ± SD (Student's *t*-test compared with DW $p > 0.05$ ns, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

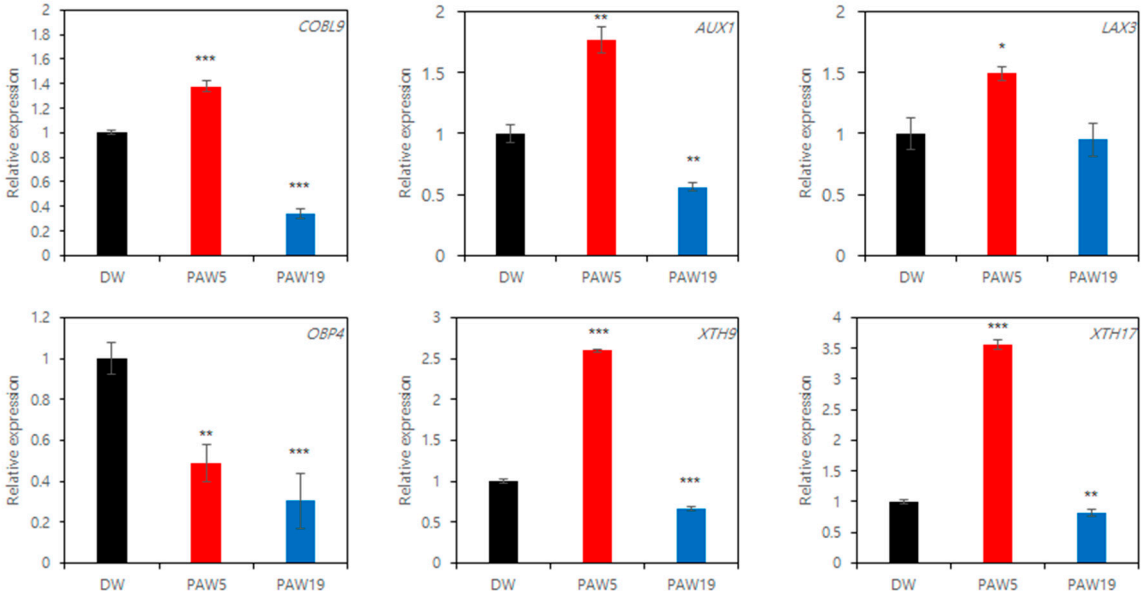


Figure 6. PAW treatment induces gene expression of root hair initiation and elongation genes in *Arabidopsis*. Student's *t*-test compared with D.W. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

4. Discussion

We investigated the effect of plasma-activated water (PAW) on the early growth and development of *Arabidopsis* in this study. The physicochemical properties of the plasma-activated water were detected according to the plasma exposure time, and the effect of the altered physicochemical properties of the PAW on plant growth was examined in *Arabidopsis*. The morphological differences in the roots and vegetative tissues, differences at the cellular level, and molecular biological differences were investigated. In addition, possible molecular mechanisms underlying the effect of the PAW treatment on

plant growth were explored in *Arabidopsis* based on the phenotype. We found that PAW facilitated the growth of the *Arabidopsis* by modulating developmental genes at an early developmental stage.

In recent years, plasma devices have emerged as an eco-friendly alternative method to improve plant growth and development. The application of plasma-related treatments in plants has been studied for improving seed germination, plant growth, plant height and yield in crops. The differences in plant phenotypes are caused by the difference in physical and chemical properties with increased biochemical components, altered seed coat morphology, biochemical activities, and altered gene expression [5–8,18]. The degree of difference in a plasma-induced plant phenotype is due to a variety and range of RONS (reactive oxygen and nitrogen species) generated by plasma with different feeder gases or just air [39]. Low pressure dielectric barrier discharge generates Ar/O_2, and plants treated with Ar/O2 produce increased H_2O_2 in the roots and shoots of wheat [40]. Cylinder double dielectric barrier discharge (DBD) reactors generate nitrate (NO_3^-) and hydrogen peroxide (H_2O_2) that positively regulate growth and germination in seeds [18,19]. Even in *Arabidopsis* as a model plant, PAW generated by air dielectric barrier discharge activates growth and development such as the leaf area, the number of flowering plants and the leaf number over a long-term observation period, and this phenomenon is also partly explained by the elevated hydrogen peroxide and nitrate [7].

In this study, the plasma generated by the SDBD device was confirmed to have a nitrogen molecule spectrum in the region of 290~400 nm (Figure 1). Nitrogen ions are mainly produced due to the plasma effect and react with H_2O. When they ionize into NO_2^- and NO_3^-, the pH decreases, and the conductivity increases. Compared to other studies on both direct and indirect plasma applications, ROS (reactive oxygen species) are the main factor affecting germination and plant growth and defense [7,40,41]. The SDBD generator device used in this study mainly produced a higher NO_3^- concentration when a longer plasma treatment time was used. In addition, the change of pH and conductivity was also significant at longer plasma treatment. This change was predicted to affect the plant growth and development. In a previous study, tobacco grown on medium containing plasma treated water had increased cotyledon growth but suppressed primary root hair growth at PAW40 [25]. *Arabidopsis* seedlings showed self-sufficient under minimal conditions for weeks without external supply of nutrients [42]. Therefore, we used the minimal media of only DW and PAW to study the more dramatic effect of the PAW treatment. By using this method, we expected to see more specific pathways that are affected by PAW. Even though it is important to study the effect of all component changes in PAW such as conductivity and osmolality, in this study we focused on the changes in the nitrate concentration which is a main nitrogen source that affects plant growth. Nitrogen is an essential macronutrient for plants and an important constituent of key molecules such as amino acids, nucleic acid and chlorophyll [43]. Nitrogen availability in the soil is a major factor in plant growth, and its availability is a key determinant of plant yield [43–46]. From this perspective, the increment of the nitrate concentration from plasma treatment potentially can serve as a very important nitrogen source for plant growth and development.

Concerning root development, the PAW treatment condition (PAW5, 7 and 12) promoted an increased total root length compared with the DW treatment condition in *Arabidopsis*; however, root development was inhibited by the PAW that was generated with a longer plasma treatment time (PAW19 and 40). In detail, the morphological differences at 15 days after planting in the root showed that the root length for the PAW5 treatment condition increased by 87%; however, it was suppressed by the PAW40 treatment condition by 63% compared to the DW control, indicating that root growth occurred independent of the plasma treatment time used to generate the PAW. Thus, *Arabidopsis* needs specific growth conditions (Figures 2 and 3). In contrast to the specific pattern of activation and inhibition of root growth, the effect of the PAW treatment in the cotyledon tissue did not show any inhibition. The cotyledon size increased according to the plasma treatment time of the PAW due to an enlarged palisade cell size, not by cell number. In

tobacco, the cotyledon size was also regulated by cell size not cell proliferation at 10 days after planting [25]. This result implies that they share the same mechanism in cotyledon development in dicotyledonous plants. In the seedlings, the roots become activated in the PAW5, 7 and 12 treatment conditions and inhibited in the PAW19 and 40 treatment conditions, and the cotyledon expands according to the plasma treatment time of the PAW as a tissue-specific plasma effect. Therefore, we need to understand it as an overall balance of the plant developmental growth and investigating the optimal treatment conditions for *Arabidopsis* is important to achieve the desired effect.

Interestingly, the PAW treatments showed an increased number of root hairs in all the PAW conditions; however, they showed a decreased root hair cell length shown by cell length measurements, in other words, a decreased trichoblast cell length (Table 2). In addition, root hair number in the *WER::GFP/cobl9-1* line increased induced by the PAW treatment while root cell elongation shortened. In the Col-0 background, the root hair number increased by 18~32% dependent on the PAW treatment time (Table 2). Root hair number in the *cobl9-1* mutant background was dramatically increased by at least two times by the PAW treatment at 5 days after planting (Table 4). It implies that the regulation of root hairs under PAW treatment involves sophisticated genetic networks, not simply a single pathway. Moreover, this result indicates the increased root hair number induced by PAW, which results in securing more space for root epidermal cells to come into contact with nutrients per unit of root cell. These differences may lead to improved growth because the root hairs are in response to the availability of NO_3^- which has an important role in nitrogen (N) source nutrient acquisition when plants are exposed to nitrate-rich soils. It is known that nitrate also can act as signaling molecules that can control downstream gene expression in plants. Previously, the change in the root architecture in plants by the nitrate concentration was presented as an example of plasticity in the developmental process in response to the environment [36,47]. For example, the increment of nitrate concentration of 0.1 to 1 mM KNO_3 was known to induce root hair and lateral root formation [48–50]. In contrast, high nitrate concentration suppressed the root formations [47,48,51].

At the molecular level, we confirmed the effect of the PAW treatment by checking the expression of marker genes related to root development. One of the key genes of root hair initiation and root hair elongations, *COBL9*, was induced in the PAW5 and suppressed in the PAW19 treatment condition. This expression result confirmed our finding of the different phenotypes in root hair number and root length between the Col-0 and *cobl9-1* mutants which were affected by the *COBL9* expression (Tables 2 and 4, Figure 6). and our PAW generated from SDBD mainly affects the nitrate concentration.

In plants, auxin has a major role in root hair development, and the auxin transporter proteins, *AUX1* and *LAX3*, regulate lateral root formation [35,50]. Short root hair is due to mutations of *AUX1* genes indicating *AUX1* controls elongation of the root hair [52]. We checked the expression of two auxin transport genes, *AUX1* and *LAX3*, which were induced in the PAW5 treatment condition; however, the expression was suppressed in the PAW19 treatment condition only in *AUX1*. This expression pattern of the *AUX1* gene is in line with a previous report that *AUX1* predominantly regulates root hair elongation whereas *LAX3* mostly regulates the lateral root formation [35]. Furthermore, we confirmed the expression of the *OBP4*, *XTH9* and *XTH17* genes that are known to regulate root hair initiation and elongation and are sensitive to the nitrate concentration in the media [50,53]. In detail, *OBP4* acts as a negative regulator of root growth, while *XTH9* and *XTH17* act as a positive regulator of root growth through cell expansion. The expression showed that these genes were affected by the PAW treatments. All the PAW treatments suppressed the expression of *OBP4*. Although the expressions of *XTH9* and *XTH17* were suppressed in the PAW19 treatment condition, the maximal observed effect was observed on the root development by the PAW5 treatment compared to all the other PAW generation times (PAW7, 12, 19 and 40). Therefore, the PAW5 condition affected the root growth by positively regulating the expression of *XTH9* and *XTH17* which may mainly be caused by the root cell expansion. Moreover, as showed in the Figure 5 and Table 5, the cell expansion of PAW19

was severely reduced, thus correlating with lower expression of *XTH* genes. It is suggested that PAW-mediated root architecture regulates the root developmental genes in *Arabidopsis*, and it is at least involved in the root auxin transporter signaling system.

Taken together, the molecular mechanism of the PAW treatment increases the nitrate concentration in PAW which induces the activation of the nitrate-responsive and cell expansion genes leading to the induction of the root hair density and root length. However, the mechanism we explored may still be affected by other characters such as a change in the osmolarity of the medium. Therefore, further detailed analyses still need to be done on each component of the PAW and their effects on plant growth. In addition, to further understand PAW regulation at the molecular level, a detailed study using current technology such as RNA-sequencing and small-RNA profiling is necessary for future applications. By revealing these mechanisms, further fine-tuned applications of plasma treatment for specific bio-samples will be elucidated from the specific target pathways in a precise manner.

5. Conclusions

PAW generated from a SDBD reactor can be used to enhance *Arabidopsis* growth in the early seedling stage. The PAW treatments were shown to affect the root hair density mediated by the *COBL9*, *XTH9* and *XTH17* genes. The maximal observed condition for *Arabidopsis* root development was identified as PAW5 in which potential target genes were up-regulated while root development was suppressed by the PAW19 treatment condition. The findings of this study will provide new insights at the molecular level into developmental stage plants and accelerate the study of applying plasma technology to crop plants.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/5/2240/s1, Supplementary Figure S1: PAW regulates number of root hair in (Col-0) Arabidopsis (a) DW, (b) PAW5, (c) PAW7, (d) PAW12, (e) PAW19. Seedlings were grown on solid media for 5 days. Bar = 100 μm. Figure S2: PAW regulates number of root hair in (WER::GFP/*cobl9-1*) *Arabidopsis* (a) DW, (b) PAW5, (c) PAW7, (d) PAW12, (e) PAW19. Seedlings were grown on solid media for 5 days. Bar = 100 μm. Table S1: List of primers used in this study.

Author Contributions: Conceptualization, Y.K.L., I.A.L., S.B.K. and R.A.P.; experiment and data curation, D.H.K., R.A.P., J.Y.P. and S.J.P.; writing—original draft preparation, D.H.K., R.A.P. and Y.K.L.; writing—review and editing, R.A.P. and Y.K.L. All authors have read and agreed to the published version of the manuscript.

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