Article

Variations in Plant Growth Characteristics due to Oxygen Plasma Irradiation on Leaf and Seed

Nobuya Hayashi [1,*], Kyotaro Yamamoto [1]

[1] Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, Fukuoka 816-8580, Japan
* Correspondence: hayashi.nobuya.056@m.kyushu-u.ac.jp

Abstract: Gene expression variations of plant leaf are investigated by irradiating seed and leaf with oxygen or air plasmas. Enhancement of leaf growth is induced by oxygen plasma irradiation on seeds, which is supported by increased gene expression for protein synthesis, oxidative-reduction reactions and decreased gene expression concerning DNA methylation and histone modification. Suppression of leaf growth is observed by the oxygen plasma, which would be owing to increased gene expression concerning heat shock protein and redox reaction, and decreased expression of photosynthesis and glycoprotein. Also, gene expression variation due to air plasma irradiation is almost same as that of oxygen plasma. Active oxygen species are major factors in both oxygen and air plasmas for the variation of gene expressions in plant.

Keywords: keyword 1; oxygen plasma 2; active oxygen species 3; plasma irradiation on seed and leaf 4; growth enhancement 5; gene expression

1. Introduction

Recently, useful effects of plants such as enhancements of germination, growth and antioxidative activity have been investigated, which are induced by active particles in plasmas [1-11]. Enhancements of root and stem lengths and leaf area have been observed [1-6]. Restriction effect of plant growth have also been observed under some plasma conditions [6]. To utilize these effects on plants and to increase producibility of agricultural products, detailed clarification of response mechanism of plants against the plasma irradiation is necessary. These growth enhancement effects would be owing to ions and active particles generated in plasmas. However, mechanism and response process of biological functions by the enhancement have not been clarified yet. Gene expression analysis of *Arabidopsis* seed irradiated with plasmas has been performed to elucidate the growth enhancement mechanism [7-9]. Observed gene expressions indicate that genes of the plant growth, stress response, hormone response, photosynthesis are upregulated. From these results, increase of the energy production pathways would occur after the germination.

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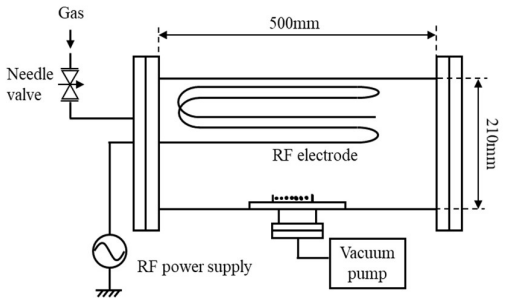

Figure 1 Schematic diagram of low-pressure plasma generation device.

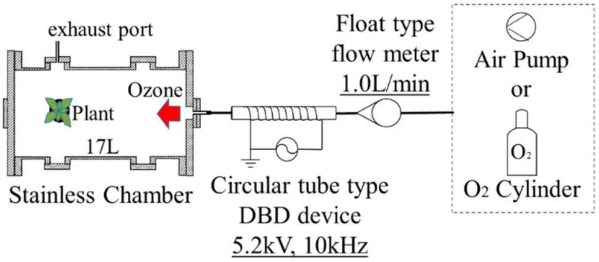

Figure 2 Schematic diagram of atmospheric plasma treatment device.

As research on the enhancement of plant growth by plasma irradiation, plasma irradiation of seeds and gene expression analysis of seeds have been mainly conducted. On the other hand, plasma irradiation to plants after germination and gene expression analysis for functional changes induced by plasma irradiation have not been performed. Gene expression in seeds is involved in germination and cell differentiation of seeds, and information on growth of sprout can be obtained [10]. To investigate the growth process of leaf, gene expression of leaf must be analyzed. In this study, to investigate the gene expression variation in leaf by irradiation seeds or leaves, both seed and leaf are irradiated with oxygen plasma, and then responses of plant leaves after germination to the oxygen plasma irradiation are determined. Effects of plasma irradiation on plant growth process are investigated by (i) gene expression of leaf that grows from seeds irradiated with plasma, (ii) gene expression of leaf that is irradiated with plasma.

2. Experimental apparatus and methods

Figure 1 shows a schematic diagram of a low-pressure capacitively coupled plasma apparatus [12-16]. The vacuum chamber is a stainless-steel cylindrical container with an inner diameter of 210 mm, a length of 480 mm, and a volume of 17 L. A high-frequency electrode is installed in the upper part of the chamber, and a sample holder on which the object to be processed is placed is installed in the diffused region. The distance between the electrode and the inner wall of the chamber is 3 cm, and the distance between the electrode and the sample holder is 17 cm. A high-frequency power supply with a frequency of 13.56 MHz and a load matching device were used for the plasma production. Plasma is produced in the gap between the high-frequency electrode and inner wall of the grounded chamber. Material gas for plasma production is pure oxygen and introduced into the chamber through the fine valve. Species and production amount of active oxygens in the plasma are measured using the light emission spectroscopy. Time variation of the temperature in the chamber is measured, because increase of the temperature in the chamber would affect gene expression of seeds. The temperature has hardly increased with plasma irradiation for 3min. After the irradiation for 30 min, After the irradiation for 30 min, the temperature around seeds increases about 10 ºC.

The torch-type dielectric barrier discharge (DBD) device is used for the plasma irradiation to leaf under atmospheric pressure [17-20]. Figure 2 illustrates the schematic diagram of plasma treatment device using the atmospheric plasma torch. A ceramics tube with dimension of 6 mm in outer diameter, 4 mm in inner diameter and 100 mm in length is used as a torch tube employing as the dielectric for discharge, and the copper film wounds on the outer surface of the ceramics tube as the grounded electrode. Cylindrical-shaped stainless-steel mesh as the cathode is set inside the ceramics tube touching on the inner wall of the tube. When the high frequency electrical power of 10 kHz is applied on the mesh electrode, the DBD occurs in the tube. Discharge voltage and current are measured using the high voltage probe and the Rogowski coil with a oscilloscope, respectively.

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The voltage and frequency applied on the cathode are set at 5.2 kV and 10 kHz, respectively. Oxygen gas or air flows into the torch tube with the flow rate of 1.0 L/min. DBD in the oxygen gas or the air at atmospheric pressure generates a plasma including ozone,

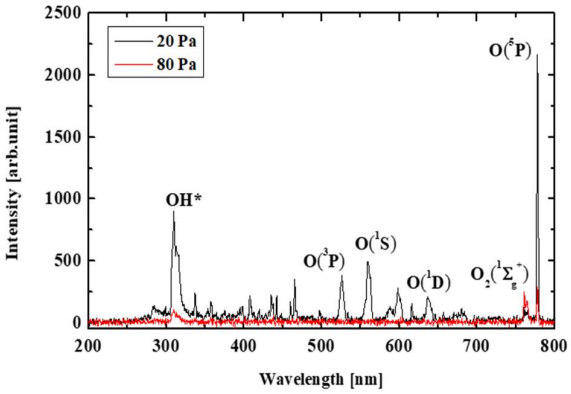

Figure 3 Typical light emission spectra of low-pressure oxygen plasma.

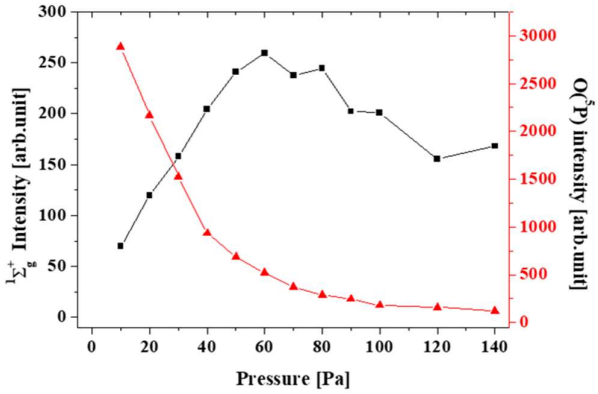

Figure 4 Light emission intensities from active oxygen species in low-pressure oxygen plasma.

and also nitrogen oxides are produced in the air plasma.

Ozone has sufficiently long lifetime to reach plants from the discharge region. In this experiment, the ozone CT value is used as the indicator of oxygen plasma effect on plants. The product of the ozone concentration C and the contact time T with ozone is defined as the ozone CT value [ppm•min] [21,22]. The concentration of ozone inside the chamber is measured by a gas detection tube using indigo dye. The ozone CT value was calculated in consideration of the fact that the ozone concentration in the chamber changes gradually with time until it is saturated, and in each case, the plasma irradiation parameters for the plants were determined so that the ozone CT value was constant. The production amount of active oxygen that is almost proportional to the CT value is measured using a chemical indicator (CI) in both cases of the low pressure and atmospheric pressure plasmas [23,24]. When active oxygen species irradiate the CI that is phthalocyanine dye pasted on a thin strip, the color of the CI changes due to oxidation of phthalocyanine. The total amount of active oxygen is quantified by the color variation using a spectrometer.

As sample plant, *Arabidopsis thaliana* wild type, Columbia-0 is supplied from the Institute of Physical and Chemical Research, RIKEN. *Arabidopsis thaliana* has been grown according to the table the guideline of plant growth of RIKEN bio resource research center. For the growth of plants, an artificial climate chamber is used to grow plants those are irradiated with the fluorescent light of 3000 Lux. *Arabidopsis thaliana* cultivated for one month after sowing seeds was placed in the stainless-steel container together with the pot and soil. After that, plasma produced by dielectric barrier discharge was introduced into the chamber. In the case of the oxygen plasma, the plant was irradiated with the plasma for 10sec and 3 times with the interval of 24 h to avoid serious ozone damage on the plant. Also, plasma irradiates seeds for 20 sec in the case of the air plasma. In both cases, the CT value of ozone is adjusted same, 1 ppm•min.

The leaf area was used as index for the growth of the plant. The area was measured from the captured images of leaves using the image processing software (Image J). The total area of 5 leaves were measured in descending order of area and evaluated using the average value. To clarify the biological reactions such as growth enhancement and anti-oxidation ability those are induced by plasma irradiation, variation of gene expression of plants after the plasma irradiation are investigated. Gene function annotation bioinformatics microarray analysis was performed as one of the gene expression analysis methods. Gene expression can be comprehensively analyzed by extracting RNA from *Arabidopsis* seeds and leaves and preparing a microarray (Arabidopsis oligo DNA microarray Ver.4.0, Agilent) in which all probes corresponding to each gene are arranged.

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Annotations of genes those are expressed due to the plasma irradiation are obtained from expressed genes with p-value less than 0.05 using the database for annotation, visualization and integrated discovery [25,26]. The protein produced can be identified from the obtained gene expression profile, and the biological reaction caused by plasma irradiation can be estimated.

3. Experimental results and discussion

3.1. Production of active oxygen species

Figure 3 shows typical light emission spectra in the diffused region of low-pressure oxygen plasma with the pressure of 20 and 80 Pa. Significant peaks are observed at 309, 762 and 777 nm in both pressure case, even though the intensity at the pressure of 80 Pa is lower than that of 20 Pa. These peaks correspond to OH radical, singlet oxygen molecule O_2 ($^1\Sigma_g^+ \rightarrow {}^3\Sigma_g^-$) and atomic oxygen O(^{5}P) [12,14,27,28], respectively. Also, peaks at 527, 559 and 636 nm those are observed at 20 Pa are attributed to atomic oxygen O(^{3}P), O(^{1}S) and O(^{1}D), respectively. These peaks cannot be observed at 80 Pa where the electron temperature decreases. Figure 4 shows the pressure dependence of light emission intensity from atomic oxygen O(^{5}P) and singlet oxygen atom O(^{1}D). The light emission intensity of O(^{5}P) decreases with oxygen gas pressure, which is due to decrease of mean electron energy from the discharge by increase of electron - neutral collisions. The excitation energy of O(^{5}P) is 5.1 eV. The number of energetic electrons decreases with the pressure, and then amount of atomic oxygen monotonically decreases. Pressure dependences of OH radical, O(^{3}P), O(^{1}S) and O(^{1}D) are almost similar with O(^{5}P). On the other hand, light emission intensity of singlet oxygen molecule O_2 ($^1\Sigma_g^+ \rightarrow {}^3\Sigma_g^-$) increases with pressure until 60 Pa, and then decreases above 60 Pa. The excitation energy of O_2 ($^1\Sigma_g^+$) is relatively lower, 1.63 eV. When the pressure is in the range of 10 to 60 Pa, production of atomic oxygens decreases and production of O_2 ($^1\Sigma_g^+$) increases. Above 60 Pa, electrons lose energy due to an increase in collisions, and the proportion of electrons that do not exceed the oxygen excitation energy increases, and then the production of O_2 ($^1\Sigma_g^+$) decreased.

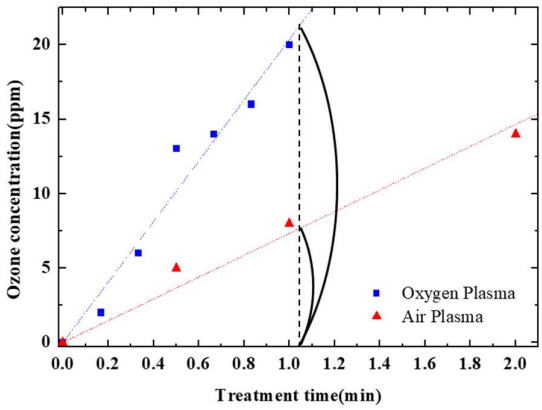

Figure 5 Ozone concentration in atmospheric plasmas changing ozone treatment time .

Atmospheric pressure plasma was generated from air or oxygen gas using dielectric barrier discharge, and was introduced into the stainless-steel chamber to control precisely the ozone CT value. Active oxygen species generated by the oxygen discharge produces the ozone in afterglow region of the atmospheric discharge. The ozone with long lifetime is transported to seed or leaf. The ozone reached the seed or leaf decomposes immediately to oxygen molecule of triplet state and singlet atomic oxygen O(^{1}D) according to the reaction equation (1) [29,30], by the catalytic action of metals in coenzyme of leaf cells:

$$O_3 \rightarrow O_2 + O(^1D) \quad (1)$$

Since atomic oxygens are difficult to be measured in general, the concentration or CT value of ozone is used as the index of atomic oxygen O(^{1}D). The concentration and irradiation time of ozone are adjusted so that CT value is 1.0 ppm•min. Short lifetime active oxygen particles such as OH radical and atomic oxygen O(^{5}P) are also generated in the plasma. The OH* and O(^{5}P) have short lifetime, and deactivate before arriving at plants. Figure 5 shows the change in ozone concentration in the chamber over plasma generation

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time. The ozone concentrations increase linearly with time. The oxygen gas discharge showed a high ozone concentration of about 2.8 times that of the air plasma.

Above results suggest that the major factor for the growth enhancement in both cases of irradiating seeds with low-pressure plasma and irradiating leaves with atmospheric-pressure plasma is singlet oxygen atom O(^{1}D). In each case of the low pressure and atmospheric pressure plasmas, amount of the active oxygen that irradiates seed and leaf is adjusted to the same using the CI due to controlling the concentration and irradiation time

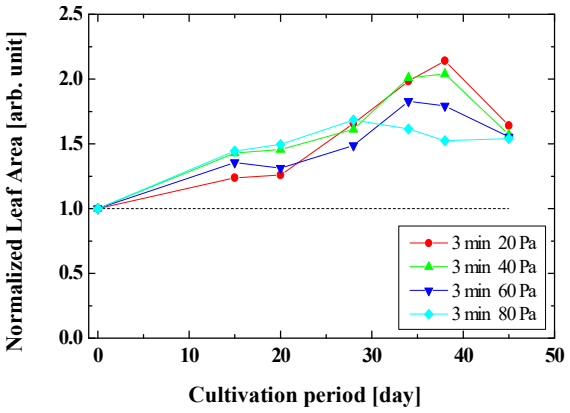

Figure 6 Cultivation period dependency of normalized leaf area by irradiation seeds with oxygen plasma for 3 min at different pressure.

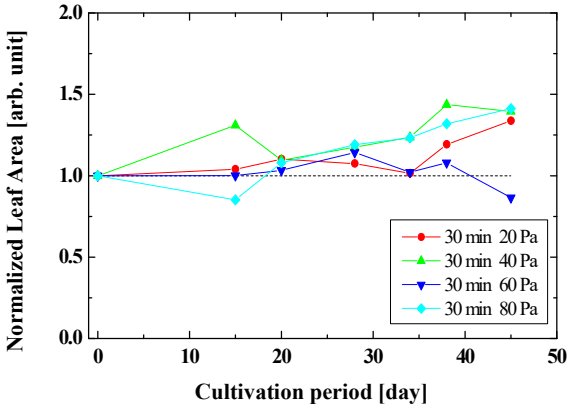

Figure 7 Cultivation period dependency of normalized leaf area by irradiation seeds with oxygen plasma for 30 min at different pressure.

of the active oxygen.

3.2. Growth enhancement characteristics of leaf by irradiating seed with oxygen plasma

Seeds of *Arabidopsis thaliana* irradiated with the low-pressure oxygen plasma for 3 minutes were seeded and cultivated. Figure 6 shows the leaf area of the *Arabidopsis* when the cultivation time is changed. The leaf area was normalized by that of unirradiated one. The leaf area became about 2.1 times after 40 days from the germination by irradiating oxygen plasma for 3 minutes with the oxygen pressure of 20 Pa as compared with that without plasma irradiation. Since the growth enhancement effect of leaves at the oxygen gas pressure of 80 Pa is almost same as that of 20 Pa, O(^{5}P) produced at any pressure would be the growth enhancement factor, and O(^{3}P) has small effect on the leaf growth enhancement. Figure 7 shows the leaf area grown from the *Arabidopsis* seeds those are irradiated with the oxygen plasma for 30 min. The leaf area becomes approximately 1.3 times after plasma irradiation for 30 min with the pressure of 60 Pa. The leaf area tends to be larger with 3 min irradiation than with 30 min irradiation. Also, there is small effect of growth enhancement at around 60 Pa. As shown in Fig. 4, light emission intensity of singlet oxygen molecule, O_2 ($^1\Sigma_g^+$) is significant at 60 Pa. Therefore, the O_2 ($^1\Sigma_g^+$) did not induce a growth enhancement effect on plants, or leaf growth was suppressed by long-term plasma irradiation for about 30 minutes. From above results, regarding the

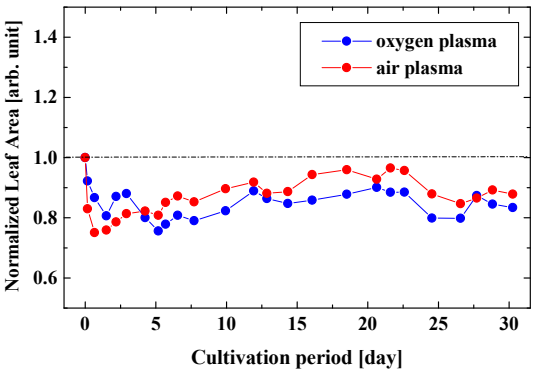

Figure 8 Cultivation period dependency of normalized leaf area irradiated with different irradiation conditions.

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growth of *Arabidopsis* leaves by irradiating seeds with the low-pressure oxygen plasma, irradiation of seeds with atomic oxygen would enhance the plant growth, and irradiation with excited oxygen molecules tends to suppress the leaf growth.

3.3. Growth enhancement characteristics of leaf by irradiating seed with oxygen plasma

Table 1 Typical annotations of expression-variable genes after irradiating seeds with oxygen plasma.

Functions and enzymes with increased gene expression	Functions and enzymes with reduced gene expression
Photosynthetic electron transport chain	Methylation
Redox reaction	DNA replication
Response to Abscisic acid	Histone

Table 2 Annotation table of increased gene expression concerning antioxidative ability by irradiating seeds with oxygen plasma.

Enrichment Score: 3.07			Count	P_Value	Benjamini
Oxidoreductase	RT		37	3.3E-5	6.9E-4
oxidation-reduction process	RT		39	6.2E-4	1.8E-2
Iron	RT		19	3.0E-2	2.1E-1
Enrichment Score: 2.01			**Count**	**P_Value**	**Benjamini**
Superoxide dismutase, copper/zinc binding domain	RT		3	1.4E-3	5.7E-2
Superoxide dismutase (Cu/Zn) / chaperones	RT		3	1.4E-3	5.7E-2
removal of superoxide radicals	RT		3	1.6E-2	2.5E-1
Copper	RT		3	3.0E-1	1.0E0

Table 3 Annotation table of increased gene expression concerning photosynthesis by irradiating seeds with oxygen plasma.

Enrichment Score: 9.03			Count	P_Value	Benjamini
pigment binding	RT		13	3.8E-17	1.1E-14
photosynthesis, light harvesting in photosystem I	RT		13	1.7E-16	7.4E-14
Photosynthesis - antenna proteins	RT		13	3.8E-16	2.4E-14
Chlorophyll a/b binding protein domain	RT		13	5.6E-16	2.5E-13
Chlorophyll A-B binding protein, plant	RT		12	1.3E-15	2.7E-13
Chlorophyll A-B binding protein	RT		13	1.8E-15	2.7E-13
Chlorophyll	RT		13	7.5E-15	1.5E-12
photosystem I	RT		13	2.8E-14	2.3E-12
light-harvesting complex	RT		11	5.6E-14	2.3E-12
Chromophore	RT		14	8.7E-14	8.9E-12
chlorophyll binding	RT		13	1.9E-13	2.8E-11
protein-chromophore linkage	RT		14	2.2E-13	4.8E-11
Photosystem I	RT		13	3.7E-13	2.6E-11
Photosynthesis	RT		18	6.4E-13	3.3E-11
plastoglobule	RT		15	3.4E-12	9.0E-11
photosystem II	RT		12	7.8E-12	1.6E-10
Photosystem II	RT		13	1.4E-11	5.6E-10
photosynthesis	RT		17	1.1E-9	1.5E-7
Thylakoid	RT		20	2.6E-8	8.8E-7
thylakoid	RT		17	5.9E-8	9.4E-7
chloroplast thylakoid	RT		16	3.5E-7	4.7E-6
chloroplast thylakoid membrane	RT		21	2.3E-6	2.6E-5

Arabidopsis leaf is irradiated with the oxygen plasma to investigate the growth enhancement of leaves. Area of leaves those are irradiated with atmospheric pressure plasma is shown in Fig. 8. Under all irradiation conditions, the area reduced to about 80% of that without the plasma irradiation on the 6th day after irradiation, and therefore the growth was suppressed by the plasma irradiation. This result is known as ozone damage in plants [31,32], and may cause problems especially in the plasma sterilization of agricultural products. Therefore, clarifying the details of ozone damage is indispensable for the progress of plasma sterilization technology.

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3.4. Gene expression analysis of leaves by irradiating seeds with oxygen plasma

To investigate biological reactions in plant after irradiating seeds with oxygen plasma, microarray analysis of RNA in leaf is performed. The RNA is extracted from the leaves cultivated for 4 weeks after sowing seeds those are irradiated with the low-pressure oxygen plasma, and then the gene expression variation from the control (un irradiated) are obtained. The irradiation conditions were an irradiation time of 3 minutes and 30 minutes with a gas pressure of 20 Pa where the atomic oxygen is dominant. There was no significant difference in the tendency of the gene expression variation when the irradiation time is changed, even though there is a remarkable difference in the growth enhancement effect with the irradiation time. This fact supports that the variation of gene expression is mainly caused by plasma irradiation on seeds.

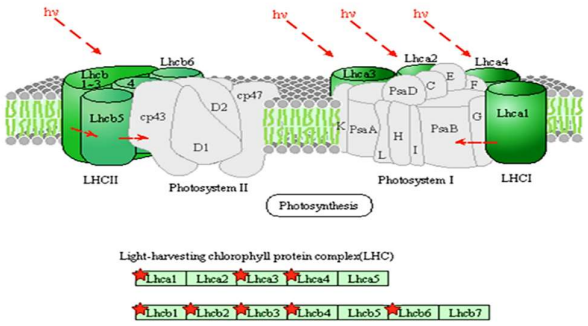

Figure 9 Signal pathway for the light-collecting chlorophyll protein complex, indicating activated reactions by oxygen plasma.

The gene expression level of the leaves irradiated with plasma for 3 minutes was compared with that of the leaves without plasma irradiation, and the expression-variable genes were extracted. Table 1 shows the gene annotations those have changed statistically significantly among expression-variable genes. The gene expression levels of (1) antioxidant activity, (2) photosynthetic electron transport chain, (3) suppression of DNA methylation, (4) molecular chaperone activation were significant for the irradiation for 30 minutes. On the other hand, expression of genes related to (5) DNA replication was reduced. The following describes the detailed function of the gene whose expression variation was significantly increased by irradiation for 3 minutes or 30 minutes:

(1) Improvement of antioxidative activity: Increased expression of the gene group was observed. By irradiating oxygen plasma for 3 minutes, as shown in Table 2, it is involved in enzymes with antioxidant activity such as oxidoreductase and superoxide dismutase (SOD). CuZn-SOD acts as a catalyst for the removal of active oxygen in plant leaf greens [33]. It is inferred that the increase of these enzymes due to the variation in gene expression enhances the sugar production by photosynthesis and the promotion of growth by removing active oxygen in cells and blocking the inhibition of dark reaction. From the above, it was found that the improvement of antioxidant activity occurs over a long period of time by changing the gene expression by short-time oxygen plasma irradiation.

(2) Enhancement of photosynthesis: From the expression analysis results, it was found that the expression of genes related to the photosynthetic electron transport chain, including chlorophyll was significantly increased by irradiation for 3 minutes, as shown in Table 3. The photosynthetic reaction obtains energy from light and produces organic molecules such as ATP from carbon dioxide and water in the atmosphere. Some enzymes involved in the Calvin cycle have one or more sets of -SH groups (thiol groups) that respond to redox, and when these are oxidized, they become -S-S- bonds (disulfide bonds). Enzymes with disulfide bonds are known to have reduced activity. Antioxidants induced by short-lifetime active oxygen irradiation may reduce the enzymes involved in the Calvin cycle and enhance the carbon fixation reaction. Figure 9 shows the pathway for the light-collecting chlorophyll protein complex of green plants whose gene expression was significantly increased by irradiation seeds with active oxygen species in the oxygen plasma for 3minutes. It is considered that the plasma irradiation for a relatively short time, 3 minutes enhanced the function of photosynthesis by increasing the expression of some genes, and promoted the production of energy such as sugar and ATP essential for protein synthesis

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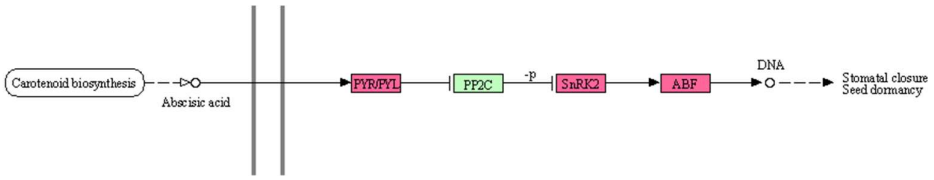

Figure 10 Signal pathway for response to abscisic acid synthesis, indicating activated reactions by oxygen plasma.

and then plant growth. On the other hand, when the seeds were irradiated with oxygen plasma for a long time, 30 minutes, there was no significant difference in the function of expressed genes, which was significantly different from that of the leaves irradiated for 3 minutes. There are two possibilities for the lack of growth-enhancement effect after 30-minute irradiation: (i) direct non-gene-mediated damage by active oxygen species to the seeds, or (ii) the Calvin cycle catalytic enzyme is inactivated by being oxidized by plasma irradiation, the Calvin cycle is suppressed and the growth of leaves is suppressed.

(3) Suppression of DNA methylation: Gene concerning DNA methylation and epigenetics are found to be expressed by irradiation with the oxygen plasma [7-9]. The plasma irradiation for 3 minutes reduced the expression level of genes related to DNA methylation. Transcription and translation of the methylated portion of DNA is suppressed. Details of the observed epigenetic gene expression induced by the plasma irradiation is described in the previous papers [7-9].

(4) Activation of molecular chaperone: Hsp90 had a high gene expression level after 30 minutes of irradiation. Hsp90 is a molecular chaperone that interacts with various proteins, has a function of preventing protein aggregation during stress, and at the same time plays an important role in signal transduction [34,35]. The increased expression of HSP suggests that plasma irradiation caused abnormalities in the protein biosynthesis system, and that the growth-enhancement effect was suppressed compared to 3-minute irradiation.

(5) Decrease of DNA replication: The rate of cell division in the leaves decreases. On the other hand, since the leaf area was expanded, it is considered that this area expansion is not due to the increase in the rate of cell division but to the cell elongation. However, no increase in gene expression related to cell elongation could be confirmed. From these facts, it is possible that the expansion of the leaf area is due to the increase in turgor pressure of the cells due to the storage of water. If the photosynthetic function is improved, it may require a large amount of H_2O. In addition, the pores used for gas exchange with the atmosphere, which are essential for photosynthesis, close to suppress transpiration when the turgor pressure is low. Thus, storing water and keeping the turgor pressure high is necessary for efficient photosynthesis. In addition, as shown in Figure 10, the gene expression level of the response to abscisic acid (ABA), which is one of the plant hormones, was increased. Expression of the response to ABA increases in response to drought stress, and ABA contributes to the efficient use of water by closing the pores and suppressing the evaporation of water [36]. It is speculated that the response to abscisic acid increased the area by storing the water.

In the previous study, the seeds were irradiated with oxygen plasma and the gene expression of the seeds was analyzed. It was confirmed that genes that upwardly control each function of growth, stress response, hormone response, and photosynthesis were expressed. This leads to the enhancement of the energy production pathway [6,8]. Similarly, in the results of gene expression analysis of leaves in this experiment, an increase in gene expression related to stress responses such as photosynthetic function and antioxidant activity was confirmed. In addition, changes in gene expression related to epigenetics such as DNA methylation were confirmed in the same way as seeds. From this result, it was suggested that oxygen plasma irradiation of seeds affected gene expression even in the grown leaves and brought about a growth enhancement effect.

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From the above results, the pathway by which plasma irradiation causes leaf growth enhancement can be inferred as follows. In the case of irradiation for 3 minutes, the gene expression of seeds subjected to oxidative stress due to active oxygen in plasma changed to improve antioxidant activity and to suppress DNA methylation in leaves. Signals related to antioxidant activity are retained even after differentiation from seed to leaf. It is considered that the enzyme that catalyzes photosynthesis is reduced and activated by the improvement of the antioxidant activity. Also, the production of NADPH, which is a reducing agent, is increased and then the photosynthetic electron transfer reaction function would be enhanced. And genes concerning water storage to improve photosynthetic efficiency. From the above, it is inferred that the efficiency of photosynthesis and the increase in ATP synthesis led to the enhancement of leaf growth. One of reaction sequences from plasma irradiation seed to leaf growth enhancement is as follows:

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1. Plasma irradiation of seeds
2. Seeds are subjected to oxidative stress and improve antioxidant activity
3. Obtained functions remains after germination (methylation: epigenetics)
4. Improvement of leaf antioxidant activity
5. Increase chlorophyll and activate photosynthetic electron transfer reaction
6. Increase water storage to improve photosynthetic efficiency, adjust circadian rhythm
7. Increase ATP synthesis
8. Promote growth (increase in leaf area).

According to the gene expression variation results, the plasma irradiation for 30 minute is expected to have the same growth enhancement effect as the 3-minute irradiation, on the other hand, the Calvin cycle is suppressed by long-term irradiation and the protein denaturation tends to occur. Therefore, the growth promoting effect would not be obtained.

3.5. Gene expression analysis of leaves irradiated with atmospheric pressure plasma

Genes whose expression varied were extracted in comparison with *Arabidopsis thaliana* unirradiated with plasma. Among these expression-variable genes, the gene annotations judged to be statistically significantly changed ($p < 0.05$) are shown in Table 4 of the annotation clustering. Increased expression of some genes involved in protein synthesis reactions was observed in the table. There was a marked increase in the expression of genes related to chaperone proteins and antioxidants. Chaperones act as catalysts in protein folding and perform endoplasmic reticulum-related degradation that removes abnormal proteins from the endoplasmic reticulum. Normally, chaperones remove abnormal proteins immediately, but when protein synthesis in the endoplasmic reticulum is active, the removal may not catch up and abnormal proteins may accumulate. At that time, a reaction called the endoplasmic reticulum stress response [37] that increases the production of factors such as chaperones occur.

Plasma irradiation of seeds increased the expression of some genes related to heat shock protein, which is a type of chaperone. Gene expression of heat shock proteins such as Hsp70 and Hsp90 was increased after the plasma irradiation, as shown in Table 5.

Table 4 Typical annotations of expression-variable genes after irradiating leaves with oxygen plasma.

Functions and enzymes with increased gene expression	Functions and enzymes with reduced gene expression
Heat shock protein	Photosynthesis
Redox reaction	glycoprotein
	Methylation

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Table 5 Annotation table of increased gene expression concerning heat shock proteins after irradiating leaves with oxygen plasma.

Enrichment Score: 2.76			Count	P_Value	Benjamini
Heat shock protein 70, conserved site	RT		7	5.6E-8	2.8E-5
Heat shock protein 70 family	RT		7	2.9E-7	4.8E-5
response to virus	RT		4	2.3E-2	3.4E-1
Enrichment Score: 2.54			**Count**	**P_Value**	**Benjamini**
Heat shock protein Hsp90, N-terminal	RT		4	1.2E-4	1.2E-2
Heat shock protein Hsp90, conserved site	RT		4	1.2E-4	1.2E-2
Heat shock protein Hsp90	RT		4	1.2E-4	1.2E-2
heat shock protein, HSP90/HTPG types	RT		4	1.4E-4	2.1E-3
Ribosomal protein S5 domain 2-type fold	RT		5	2.3E-2	4.9E-1
Histidine kinase-like ATPase, ATP-binding domain	RT		4	2.5E-2	5.0E-1
HATPase_c	RT		3	3.4E-2	6.9E-1
Plant-pathogen interaction	RT		8	3.6E-2	2.5E-1

Table 6 Annotation table of increased gene expression concerning redox reactions after irradiating leaves with oxygen plasma.

Enrichment Score: 3.03			Count	P_Value	Benjamini
response to endoplasmic reticulum stress	RT		7	3.9E-7	8.7E-5
Redox-active center	RT		12	3.1E-6	1.6E-4
Disulphide isomerase	RT		4	7.0E-5	8.5E-3
protein disulfide isomerase activity	RT		5	8.4E-4	1.2E-1
domain:Thioredoxin 1	RT		3	8.5E-4	4.3E-2
site:Lowers pKa of C-terminal Cys of second active site	RT		3	8.5E-4	4.3E-2
site:Lowers pKa of C-terminal Cys of first active site	RT		3	8.5E-4	4.3E-2
domain:Thioredoxin 2	RT		3	8.5E-4	4.3E-2
Protein disulphide isomerase	RT		3	1.4E-3	9.3E-2
Thioredoxin, conserved site	RT		5	1.6E-3	9.3E-2
Thioredoxin domain	RT		7	2.1E-3	1.1E-1
cell redox homeostasis	RT		9	2.7E-3	1.0E-1
Thioredoxin-like fold	RT		11	5.4E-3	2.2E-1

Hsp70 covers a wide range of proteins such as folding and association of nascent proteins, inhibition and repair of aggregation of proteins partially decomposed by stress such as heat, transport of proteins to cell organs, regulation of cell division and transcription, and regulation of protein activity. Hsp90 is a molecular chaperone that plays a role in preventing protein aggregation during stress, as described above. From the whole pathway of protein synthesis in endoplasmic reticulum, the expression of genes involved in the mechanism for detecting abnormal proteins (Protein Recognition by luminal chaperon) and the mechanism for removing abnormal proteins (ERAD) significantly increased by the plasma irradiation. In leaf cells, while atmospheric pressure plasma irradiation produced a growth-promoting effect, the growth would be suppressed as a result of damage such as protein denaturation. It is presumed that protein synthesis in the endoplasmic reticulum became active due to the recovery of the damaged site and the increase in resistance, and the molecular chaperone production improved in response to the accompanying accumulation of abnormal proteins those are generated by the plasma irradiation.

Table 6 shows a cluster of gene expression related to the redox reaction in which the expression variation was increased by the plasma irradiation leaves. Thioredoxin is a protein that functions as antioxidant that reduces disulfide bonds and promotes conversion to thiol groups [38,39]. As mentioned above, some enzymes involved in the carbon fixation reaction of photosynthesis, such as RubisCO, are activated by being reduced by antioxidative substances like Thioredoxin. Protein disulphide isomerase (PDI) is a protein with a Thioredoxin-like structure that catalyzes the thiol-disulfide exchange reaction of proteins and assists in the folding of proteins [40,41]. From the increase in the expression of these genes, it is inferred that proteins concerning the redox reactions in plants were

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Table 7 Annotation table of reduced gene expression concerning photosynthesis after irradiating leaves with oxygen plasma.

Enrichment Score: 9.03			Count	P_Value	Benjamini
pigment binding	RT		13	3.8E-17	1.1E-14
photosynthesis, light harvesting in photosystem I	RT		13	1.7E-16	7.4E-14
Photosynthesis - antenna proteins	RT		13	3.8E-16	2.4E-14
Chlorophyll a/b binding protein domain	RT		13	5.6E-16	2.5E-13
Chlorophyll A-B binding protein, plant	RT		12	1.3E-15	2.7E-13
Chlorophyll A-B binding protein	RT		13	1.8E-15	2.7E-13
Chlorophyll	RT		13	7.5E-15	1.5E-12
photosystem I	RT		13	2.8E-14	2.3E-12
light-harvesting complex	RT		11	5.6E-14	2.3E-12
Chromophore	RT		14	8.7E-14	8.9E-12
chlorophyll binding	RT		13	1.9E-13	2.8E-11
protein-chromophore linkage	RT		14	2.2E-13	4.8E-11
Photosystem I	RT		13	3.7E-13	2.6E-11
Photosynthesis	RT		18	6.4E-13	3.3E-11
plastoglobule	RT		15	3.4E-12	9.0E-11
photosystem II	RT		12	7.8E-12	1.6E-10
Photosystem II	RT		13	1.4E-11	5.6E-10
photosynthesis	RT		17	1.1E-9	1.5E-7
Thylakoid	RT		20	2.6E-8	8.8E-7
thylakoid	RT		17	5.9E-8	9.4E-7
chloroplast thylakoid	RT		16	3.5E-7	4.7E-6
chloroplast thylakoid membrane	RT		21	2.3E-6	2.6E-5

Table 8 Annotation table of reduced gene expression concerning glycoproteins after irradiating leaves with oxygen plasma.

Enrichment Score: 5.75			Count	P_Value	Benjamini
Signal	RT		150	1.4E-11	3.2E-9
extracellular region	RT		99	3.2E-6	2.0E-4
Glycoprotein	RT		71	7.7E-6	9.0E-4
signal peptide	RT		67	1.0E-5	5.4E-3
Secreted	RT		54	4.2E-5	3.3E-3
glycosylation site:N-linked (GlcNAc...)	RT		46	2.1E-4	5.4E-2

oxidized by the plasma irradiation. It is inferred that the active species in the plasma have reached the inside of the plant.

Genes with reduced gene expression are investigated. Table 7 shows the cluster of gene expression related to photosynthesis in which gene expression was reduced. The gene expression concerning the photosynthetic electron transfer reaction function was significantly reduced in this experiment. The gene expression level of the light-harvesting chlorophyll protein complex (LHC) containing chlorophyll was decreased by plasma irradiation [42]. Table 8 shows the cluster of gene expression for glycoproteins whose gene expression was reduced. In many cases, oligosaccharides covalently bind to proteins that enter the endoplasmic reticulum cavity or endoplasmic reticulum membrane to become glycoproteins. It is suggested that the production amount of sugar produced decreased because the amount of ATP, which is the energy in the body, decreased due to the impaired photosynthetic function, and the production of glycoprotein was also suppressed accordingly. One of reaction sequences from plasma irradiation leaf to growth enhancement of leaf is as follows:

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1. Plasma irradiation of leaves
2. Damage to the photosystem antenna complex due to oxidative stress
3. Decrease in reaction rate of photosynthetic electron transfer reaction
4. Increased production of chaperone for protein repair
5. Decrease of ATP and sugar synthesis amount due to carbon fixation reaction
6. Protein decrease in biosynthesis

7. Promote growth (increase in leaf area)

Table 9 Typical annotations of expression-variable genes after irradiating leaves with air plasma.

Functions and enzymes with increased gene expression	Functions and enzymes with reduced gene expression
Biodefense reaction	Glycoprotein
Redox reaction	Growth factor
	Protein phosphorylation

To investigate the effect of nitrogen species on the gene expression variations of the plant, atmospheric air discharge plasma is employed to irradiate the plant leaves. Table 9 shows gene annotations those have statistically significantly changed between the expressed genes in *Arabidopsis* leaves those had been exposed to the atmospheric air plasma for 30 seconds and the genes of the unirradiated leaves. The ozone CT values of the atmospheric oxygen and air plasmas are same, 1.0 ppm•min, to reveal the effect of active nitrogen species in the air plasma. There was almost no significant difference between the air and the oxygen plasmas in the function of the expressed gene that was significantly varied compared to the atmospheric oxygen plasma case. Also, the main reaction pathways of the plant for the air and the oxygen plasmas are almost similar. Compared with oxygen plasma, nitrogen-based active species are produced in air plasma, but the influence of oxygen-active species is large on plants.

3.6. Comparison of leaf gene expression variation analysis before and after germination

The gene expression analysis of the leaves that were plasma-irradiated to the seeds before germination is compared with the leaves that were plasma-irradiated to seeds after germination. Gene expression related to photosynthetic function increased when plasma irradiation was performed before germination, but decreased when plasma irradiation was performed after germination. Gene expression related to stress response such as antioxidant activity tended to increase under both conditions. From these facts, it is speculated that it is possible to improve the resistance of leaves to oxidative stress after germination by irradiating them with plasma before germination. If it has resistance to oxidative stress due to plasma irradiation, it may be applicable to agricultural product sterilization with ozone.

4. Conclusion

Plasma irradiation was performed on Arabidopsis seeds and leaves to investigate the mechanism of plant growth enhancement and to clarify the effect of plasma irradiation during the growth process. Gene expression analysis was performed on the leaves grown from the seeds irradiated with plasma and the leaves irradiated with plasma after germination. The list of the obtained results is described below:

1. The leaf area of Arabidopsis promotes growth up to 2.1 times by irradiation of seeds with low-pressure oxygen plasma. On the other hand, plasma irradiation of the leaves suppressed the growth about 0.8 times.
2. Plasma irradiation of seeds significantly increased gene expression related to leaf photosynthesis, antioxidant activity and water storage. From this, it is presumed that the production of NADPH was increased by photosynthesis as the oxidation resistance to plasma irradiation, and the water storage was improved accordingly, leading to the promotion of growth.
3. Comparing the results of gene expression analysis of seeds and leaves after plasma irradiation of seeds, it was confirmed that both seeds and leaves had increased gene expression related to the same function such as photosynthesis. This suggests that plasma

irradiation of seeds also affects leaf gene expression, and it is considered that this phenomenon is related to epigenetic changes such as methylation.
4. Plasma irradiation of leaves reduced gene expression related to leaf photosynthesis. On the other hand, the antioxidant activity of the leaves increased.

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