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Effect of Oxygen Plasma Irradiation on Gene Expression in Plant Seeds Induced by Active Oxygen Species

Riku Nakano,[a,*] Kosuke Tashiro,[b] Reona Aijima,[c] & Nobuya Hayashi[a]

[a]Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, Fukuoka 816-8580, Japan; [b]Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; [c]Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Saga University, Saga 849-8501, Japan

*Address all correspondence to: Riku Nakano, Interdisciplinary Graduate School of Engineering Sciences, Kyushu University, 6-1 Kasuga Koen, Kasuga, Fukuoka 816-8580, Japan; Tel.: +81-92-583-7651; Fax: +81-92-571-8894, E-mail: r.nakano@aees.kyushu-u.ac.jp

ABSTRACT: We investigated the mechanism of gene expression in plant seeds induced by low-pressure oxygen radio frequency plasma irradiation using microarray analysis and quantification of DNA methylation. A decrease in the amount of gene expression of *AT2G30620* indicated that the chromatin structure is modified by active oxygen species generated in oxygen plasma. The increase in the DNA methylation level in plant seeds was determined by using a DNA methylation quantification method. Gene expression in plant seeds induced by active oxygen species was influenced by the epigenetic variation of DNA methylation and chromatin structure.

KEY WORDS: oxygen plasma, active oxygen species, plant growth enhancement, epigenetics, DNA methylation

I. INTRODUCTION

Growth enhancement of plants that is induced by active oxygen species produced in oxygen plasma has a major effect on plasma stimulation to plants.[1–7] Living bodies produce and use active oxygen species for chemical reactions or signal transduction, and the balance of active species is highly regulated.[8–12] When oxygen plasma irradiates a living body, the redox balance inside the cell changes slightly, and counterreactions occur to maintain the redox balance of the cell. To induce these counterreactions in cells, a living body varies its gene expression. For example, when seeds are irradiated by active oxygen species in oxygen plasma, the plasma-induced redox reactions initiate photosynthesis and energy production in plants, a phenomenon of plant growth enhancement. Some characteristics of plasma-induced plant growth enhancement effects have been clarified. First, the growth enhancement lasts for the plant's entire lifetime, and the rate of plant length growth is higher than that of a control in the first generation. Second, the growth enhancement effect is not passed onto plants in the next generation. When seeds of second generation are cropped from plants irradiated by oxygen plasma in the first generation and then cultivated, the growth speed is almost same as that of the control.

These facts indicate that growth enhancement is inherited by daughter cells through cell division within the first generation.[1] Thus, the nuclear DNA sequence has not been modified and genetic mutations are not generated by the active oxygen species in the oxygen plasma.

Although active oxygen species produced in oxygen plasma do not modify the DNA sequence, plant growth enhancement as a result of gene expression due to active oxygen species has been observed when seeds of radish sprouts are irradiated by oxygen plasma. However, the mechanism of the effects of active oxygen species on plant seeds, which induce plant growth enhancement without modifying the DNA sequence, has not been clarified. In this study, we investigated the mechanism of gene expression induced by active oxygen species in oxygen plasma as a stimulus to plants using microarray analysis and DNA methylation quantification analysis.

II. EXPERIMENTAL APPARATUS AND METHOD

Figure 1 shows a schematic diagram of the experimental apparatus. The capacity of the chamber was 20 L. A radio frequency (RF) electrode was set inside the vacuum chamber along the chamber wall. The electrode shape was that of a wound rod, with a total length of 200 cm. We set the RF power of 13.56 MHz to 60 W throughout the experiment and gas pressure to 20, 40, and 80 Pa. The material gas was argon or oxygen. We used *Arabidopsis thaliana* (wild type) and *Raphanus sativus* (radish sprouts) as model plants for this study. The plant seeds were enclosed in a nonwoven bag to avoid ion impact and placed at the bottom of the chamber. We varied the plasma treatment period for each set

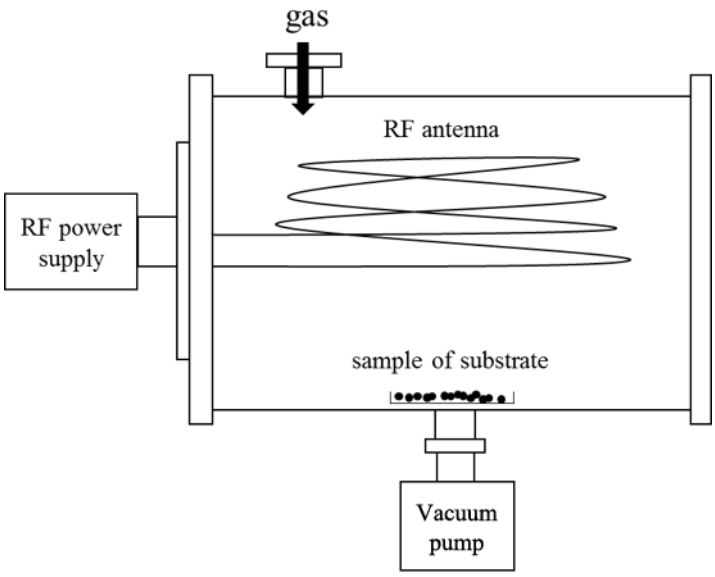

FIG. 1: Schematic diagram of the experimental apparatus

of conditions. The temperature of the seeds during plasma irradiation was maintained to be at least 10°C higher than room temperature.

To manufacture a microarray for gene analysis, we extracted RNA from 50 seeds using an extraction reagent after *Arabidopsis* seeds were irradiated by an oxygen plasma. The quality of the extracted RNA was confirmed to be sufficient for microarray analysis by electrophoresis. We determined variation in gene expression of the seed RNA irradiated by the oxygen plasma from the microarray using a microarray scanner (Agilent SurePrint G3GE 8x60K, version 2, Agilent Techonologies, Inc., California, USA).[13] We sorted the gene expression chart that we obtained by the function of the genes using a functional annotation bioinformatics microarray method and pathway analysis for *Arabidopsis* using a database for annotation, visualization, and integrated discovery (DAVID).[13] To determine the level of DNA methylation as a marker of epigenetics, we extracted *Arabidopsis* DNA (100 ng) from seeds using a DNA extraction reagent (DNeasy plant mini kit, Qiajen, Inc,. Hilden, Germany) and quantified the 5-methylcytosine in the extracted DNA using a DNA methylation quantification kit.

III. RESULT AND DISCUSSION

A. Active Species Generated in Oxygen Plasma

Figure 2 shows optical emission spectra of oxygen plasma measured in the afterglow region 120 mm below the RF electrode, where seeds to be treated were placed. Peaks at 527, 558, 596, 636, 762, and 777 nm were assigned to atomic oxygen $O(^3D)$, $O(^1\Sigma)$, $O(^3D^1)$, $O(^1D)$, the oxygen molecule ($^1\Sigma_g^+$), and $O(^5P)$, respectively. Langmuir probe

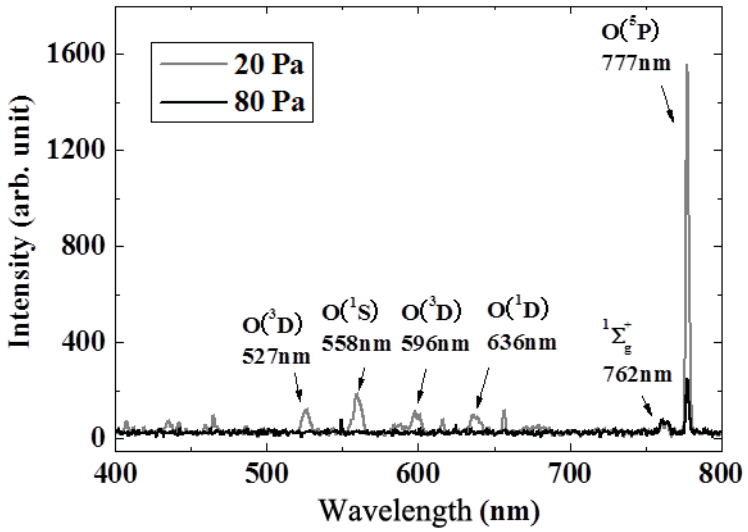

FIG. 2: Light emission spectra of oxygen plasma at gas pressures of 20 and 80 Pa

characteristics indicated that the charged particle density in the afterglow region was $<10^{12}$ m^{-3}, which is the lower detection limit of the Langmuir probe. Therefore, the dominant species in the afterglow region of the oxygen plasma were active oxygen species. The oxidation energies of these active oxygen species were <5 eV, insufficient to modify base sequences in DNA. Therefore, genetic mutations could not occur from irradiation by an oxygen RF plasma. Figure 3 shows the light emission intensity of $^1\Sigma_g^+$ and O(^{5}P) at varying gas pressures. Production of atomic oxygen requires electrons with higher energy, and the intensity of O(^{5}P) increased with decreasing pressure. However, the amount of $^1\Sigma_g^+$ had a peak at ~40 Pa, because production of an excited molecule requires moderate particle energy to avoid dissociation of the molecule. When the oxygen gas pressure increases, the ratio of the amount of $^1\Sigma_g^+$ to O(^{5}P), which indicates the relative effectiveness or affectivity of $^1\Sigma_g^+$ out of $^1\Sigma_g^+$ to O(^{5}P), increases monotonically, as indicated by the open circles in Fig. 3. The ratio of $^1\Sigma_g^+$ to O(^{5}P) increases with the oxygen gas pressure. As mentioned above, oxygen gas pressures of 20, 40, and 80 Pa were chosen as the plasma conditions for the following experiments.

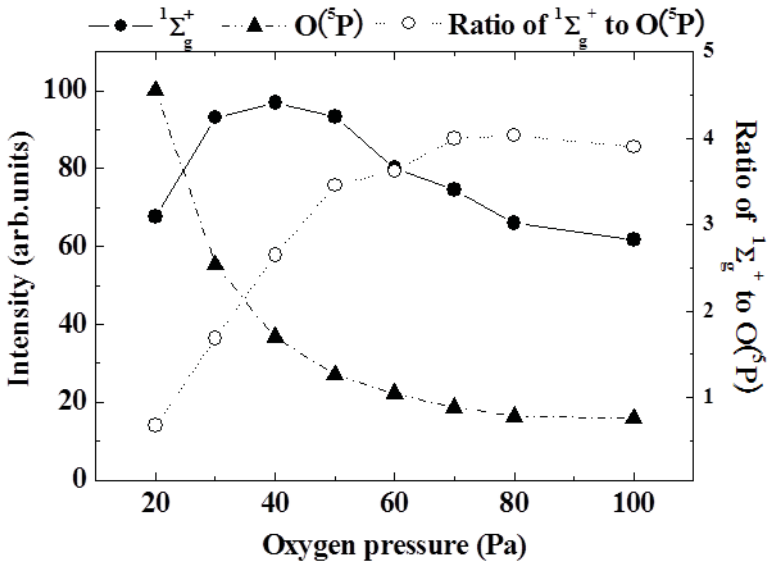

FIG. 3: Dependence of light emission intensities of active oxygen molecules and atomic oxygen on oxygen pressure

B. Plasma Irradiation Effect on Seeds

Figure 4 shows typical results of growth enhancement by plasma stimulation. As mentioned above, radish sprouts, used here, were one of the plants adopted as a model plant in this experiment. Figure 4(a) shows the normalized plant length varying with the plas-

ma treatment period. Gas pressure was kept at 80 Pa. Plant length with 170% of the control value was obtained at the plasma treatment period of 15 min. Figure 4(b) shows the results of plant length normalized to the control value with varying gas pressure. The treatment period was set to 30 min. Growth enhancement increased with increasing gas pressure from 20 to 80 Pa. The emission intensity of atomic oxygen O(^{5}P) shown in Fig.

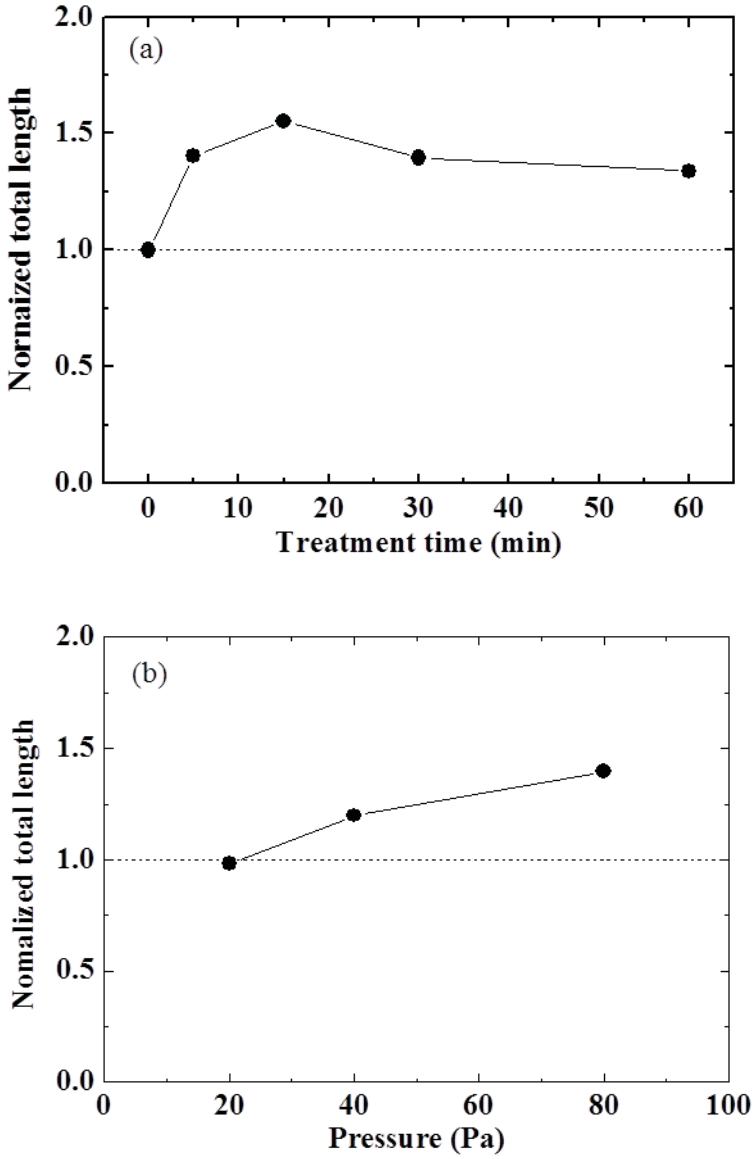

FIG. 4: Dependencies of normalized plant lengths irradiated by oxygen plasma on the (a) plasma treatment period and (b) oxygen pressure

3 and the plant growth enhancement effect shown in Fig. 4(b) are correlated in opposite directions. Considering the ratio of the amount of $^1\Sigma_g^+$ to O(^{5}P) at varying gas pressures, the observed pressure dependence of plant growth showed the same tendency as that of the contribution of excited oxygen molecules. Therefore, an active oxygen species with a long lifetime, $^1\Sigma_g^+$, contributed to growth enhancement. Because the effect of plant growth enhancement is not induced by DNA mutation, the induction of the growth enhancement effect by active oxygen species is likely a result of epigenetics.

C. Plasma-Induced Gene Expression

1. Microarray Analysis

Active oxygen species induce many chemical reactions in seeds related to oxidative stress or kinetic reactions, which leads to changes in gene expression. To investigate the variation in gene expression, microarray analysis was performed, and then gene ontology (GO) analysis was used to extract the associated biological processes using DAVID. Genes used for GO analysis were chosen with the criteria that the ratio of gene expression must be >1.5, with a derivation value of >2. Figure 5 shows a functional annotation chart of biological processes related to plant growth. In Fig. 5, genes listed in the functional annotation chart as having variation in expression have p values <0.05. Based on microarray analysis, the expression of many genes changed. These variations were not due to genetic mutation, because growth enhancement is not inherited by the second-generation plants. Therefore, the list of all genes showing changes in expression that are obtained from microarray analysis is important, because it reflects information about the epigenetic processes affected by active oxygen species, not genetic mutations.

Some of the obtained GO terms indicate that the level of gene expression related to metabolic enzymes changed significantly. For example, the pathway of starch and sucrose metabolism was up-regulated, which led to an increase in ATP production. These biological processes, such as starch and sucrose metabolism, are up-regulated by stimulation of active oxygen species. These processes would lead to growth enhancement as a counterreaction to plasma irradiation. On the other hand, biological processes related to structure in chromosome regions were activated, as shown in Fig. 5. This indicates that the structure of chromosome regions, such as chromatin, nucleosomes, and DNA, is modified by stimulation of active oxygen species. These processes are speculated to lead to changes in gene expression due to epigenetic modification. We then investigated characteristics of the expressed genes.

To confirm epigenetic phenomena caused by plasma irradiation of plant seeds, microarray analysis identified genes related to the structure of chromosome regions that showed the same tendency for changes in level of gene expression under each set of conditions. Figure 6 shows normalized expression of two genes, *AT2G30620* and *RNA-directed DNA methylation 4* (*RDM4*), for different gas species and gas pressures. *AT2G30620* encodes histone H1.2, a protein related to chromatin structure. The linker

GO term related to plant growth	Count	P-Value
response to wounding	13	4.68E-05
cell wall organization	18	5.41E-05
external encapsulating structure organization	5	1.07E-04
nitrate assimilation	5	2.52E-04
nitrate metabolic process	4	2.52E-04
response to nitrate	10	3.17E-03
unidimensional cell growth	11	4.67E-03
cell morphogenesis	8	1.03E-02
developmental growth	11	1.15E-02
cellular component morphogenesis	10	2.01E-02
cell growth	8	3.14E-02
fatty acid biosynthetic process	5	4.42E-02
cell tip growth	6	4.97E-02
cell morphogenesis involved in differentiation	10	5.34E-02
regulation of cellular component size	8	5.51E-02
sulfur metabolic process	5	6.07E-02
sulfur amino acid metabolic process	5	6.40E-02
developmental cell growth	24	6.40E-02
reproductive developmental	9	6.42E-02
fatty acid metabolic process	6	7.18E-02
one-carbon metabolic process	14	7.40E-02
organic acid biosynthetic process	14	7.45E-02
nitric oxide metabolic process	2	7.90E-02
nitric oxide biosynthetic process	2	7.90E-02
carbohydrate transport	5	8.91E-02
glycoside metabolic process	5	9.21E-02
reproductive cellular process	6	9.07E-02

GO Term related to structure in chromosome region	Count	PValue
GO:0006355~regulation of transcription, DNA-dependent	24	5.80E-05
GO:0051252~regulation of RNA metabolic process	24	6.31E-05
GO:0045449~regulation of transcription	34	2.00E-04
GO:0006350~transcription	23	2.01E-03
GO:0016570~histone modification	4	1.28E-02
GO:0016569~covalent chromatin modification	4	1.47E-02
GO:0006306~DNA methylation	3	2.97E-02
GO:0006305~DNA alkylation	3	2.97E-02
GO:0016568~chromatin modification	5	3.18E-02
GO:0006304~DNA modification	3	3.34E-02
GO:0032776~DNA methylation on cytosine	2	5.12E-02
GO:0010629~negative regulation of gene expression	5	5.62E-02

FIG. 5: Functional annotations of biological processes concerning plant growth and chromosome structure.

histone H1.2 is generally believed to be involved in chromatin organization by stabilizing chromatin structure.[14] The role of histone H1.2 protein is as a transcription repressor that prevents the access of transcription factors or DNA-binding proteins.[15] The level of expression of *AT2G30620* decreased in all plasma conditions, as shown in Fig. 6(a). This indicates that the chromatin structure is modified by stimulation with active oxygen species. The level of expression of the RDM4 protein, which codes for proteins related

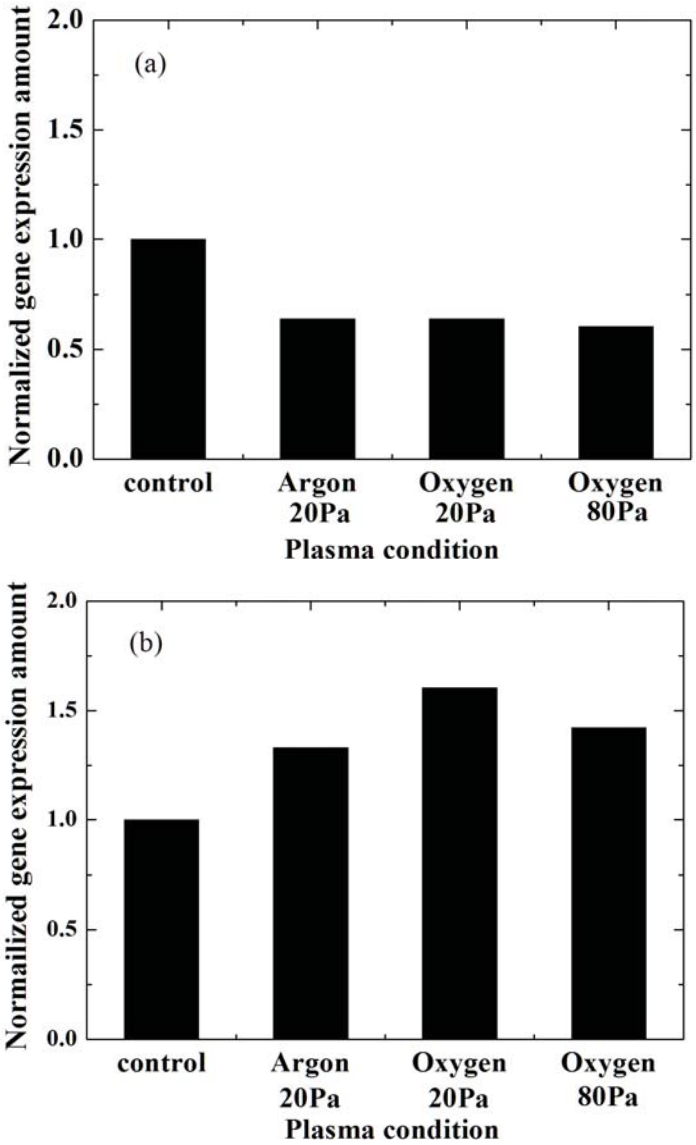

FIG. 6: Ratio of expression of genes in varying gas species and pressures. (a) *AT2G30620*, encoding histone H1.2; (b) *RDM4*, encoding a protein inducing DNA methylation

to DNA methylation, was measured. DNA methylation typically acts to repress gene transcription when a methyl group is located in a gene promoter. Expression of RDM4 leads to gene silencing.[16] Figure 6(b) shows the normalized gene expression of RDM4. The expression of RDM4 increased by 30% in an argon plasma with a gas pressure of 20 Pa. Therefore, the amount of DNA methylation is modified by the active oxygen species generated in an oxygen plasma. When argon plasma irradiates seeds, the expression of the *AT2G30620* and *RDM4* genes changed, as shown in Fig. 4, as with oxygen plasma. This observation indicates that chromosome structure modification is a result of kinetic damage to plant cells as well as oxidative stress.

2. DNA Methylation Quantification Analysis

DNA methylation was quantified to clarify epigenetic modifications by plasma irradiation. Cells in seeds were stimulated by active oxygen species generated in oxygen plasma; active oxygen species would cause oxidative stress in seeds. We set the treatment period to 30 min, and varied the oxygen gas pressure at 20, 40, and 80 Pa. In this experiment, 5-methylcytosine was measured as an indicator of DNA methylation. Figure 7 shows the amount of 5-methylcytosine and the ratio of $^1\Sigma_g^+$ and O(^{5}P) at varying oxygen gas pressures. The amount of 5-methylcytosine induced by oxygen species indicated that the amount of DNA methylation increased monotonically with increasing gas pressure, as shown in Fig. 7. The ratio of $^1\Sigma_g^+$ and O(^{5}P), which indicates the effect of $^1\Sigma_g^+$, also increased monotonically with increasing gas pressures. This figure shows that

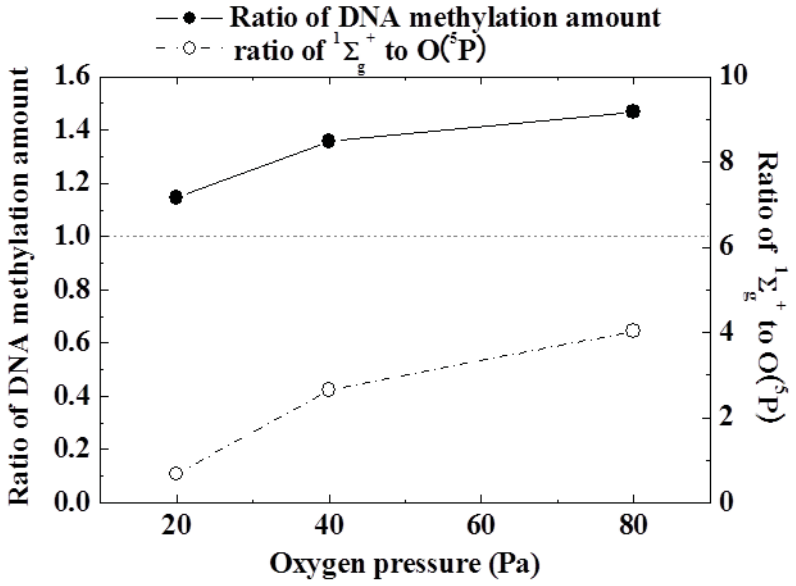

FIG. 7: DNA methylation amount and ratio of $^1\Sigma_g^+$ and O(^{5}P) varying oxygen pressure

$^1\Sigma_g^+$ is an important factor affecting DNA methylation, and $^1\Sigma_g^+$, which has moderate energy, induces DNA methylation. As shown in Fig. 4(b), the dependence of plant length on oxygen gas pressure is similar to the tendency in Fig. 7. This indicates that DNA methylation is one of the key processes involved in growth enhancement of the plants.

DNA methylation induced by active oxygen species modifies aspects of chromosome structure related to chromatin remodeling.[17,18] Remodeling of chromatin regions affects gene expression by controlling transcription factors or other DNA-binding proteins to access condensed DNA. Therefore, modification of chromatin and DNA methylation may induce variation in gene expression related to plant growth enhancement. Because DNA methylation tends to be passed on to daughter cells through cell division, stimulation by active oxygen species that induces modification of chromosome structure would be maintained in daughter cells.

IV. CONCLUSION

We investigated the mechanism of gene expression of plant seeds induced by oxygen plasma using microarray analysis and quantification of DNA methylation. Based on microarray analysis, we observed that variation in gene expression of seeds was a response to the irradiation of oxygen plasma. The amount of expression of epigenetic genes relating to the structure of chromatin and DNA methylation varied. The amount of DNA methylation in seeds increased after stimulation by active oxygen species in oxygen plasma. Therefore, it is clear that irradiation of oxygen plasma induces epigenetic modification in plant cells and caused gene expression to vary. In turn, variation of gene expression by an epigenetic process led to inducing the effect of growth enhancement.

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