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DNA Microarray Analysis of Plant Seeds Irradiated by Active Oxygen Species in Oxygen Plasma

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ABSTRACT: Gene expressions in plant seeds irradiated by oxygen plasma were investigated using DNA microarray bioinformatics analysis to clarify the pathways responsible for growth enhancement of plants. Gene expressions involved in photosynthesis and energy production by active oxygen species in oxygen plasma affect the growth enhancement of plants. The growth enhancement effect is not passed on to the next generation, and there is no significant change in gene expression in second-generation seeds by the plasma irradiation. The observed growth enhancement of plants is brought about by epigenetics.

KEY WORDS: active oxygen species, growth enhancement, microarray analysis, gene expression, reaction pathways

I. INTRODUCTION

Biological reactions of plants such as germination and growth enhancements induced by plasma irradiation using different discharge types[1–3] and various gases have been investigated.[4,5] Recently, plant germination and growth regulations has been observed when plant seeds are irradiated by active oxygen species produced by oxygen plasmas.[6–11] Some biochemical reactions that occur inside plant cells are affected by active oxygen species, and photosynthesis and/or protein production are enhanced. The first reactions induced by active oxygen species are the redox reactions of thiol compounds.[12–17] When plant seeds are irradiated by active oxygen species generated by oxygen plasma, thiol compounds in cells are oxidized, and the thiol bases change into disulfide bonds.[9, 18] The major thiol compound in living organisms is thioredoxin,[16,19] which plays roles as an oxygen sensor and a scavenger of excess active oxygen species. However, the pathways by which the oxidation of thiol compounds by exterior active oxygens affect growth regulation have not been elucidated. The oxidation and reduction of thiol compounds should lead to the regulation of enzymatic reactions, including gene expression.[12–14]

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Clarification of the involved pathways is important to confirm the safety of plasma applications for agriculture, such as the disinfection of agricultural products and sterilization of seeds. However, no definite method has been developed to clarify the mechanism of plant growth enhancement. In the present study, to propose highly reasonable mechanisms of the plant growth enhancement induced by active oxygen species, the gene expression of plant seeds was investigated by functional annotation bioinformatics analysis[20,21] and gene ontology analysis[23,24] using microarrays.

II. EXPERIMENTAL SETUP

Arabidopsis thaliana and *Raphanus sativus* (radish sprout) seeds were irradiated by active oxygen species generated in an oxygen radio-frequency (RF) plasma at low pressure,[9,11,18] as shown in Fig. 1. Oxygen gas was introduced into the vacuum chamber with a capacity of 20 L, and pressure was set at 20–80 Pa. The RF antenna was positioned inside the vacuum chamber along the chamber wall. The shape of the antenna is a wound rod with a total length of 200 cm. The RF power with 13.56 MHz frequency was kept at 60 W throughout the experiment. The oxygen plasma generated by the RF discharge localized around the RF antenna due to the difficulty of the oxygen discharge at relatively higher pressure. Active oxygen species were confirmed by light emission spectra and a chemical indicator, which were set at the position of the seeds. The chemical indicator used in this experiment was designed for the detection of neutral active oxygen species.[22] The seeds were enclosed in a nonwoven bag to avoid stimulation by ion impacts and were placed at the bottom of the chamber.

In this experiment, radish sprout and *Arabidopsis thaliana* (wild type, Columbia-01)

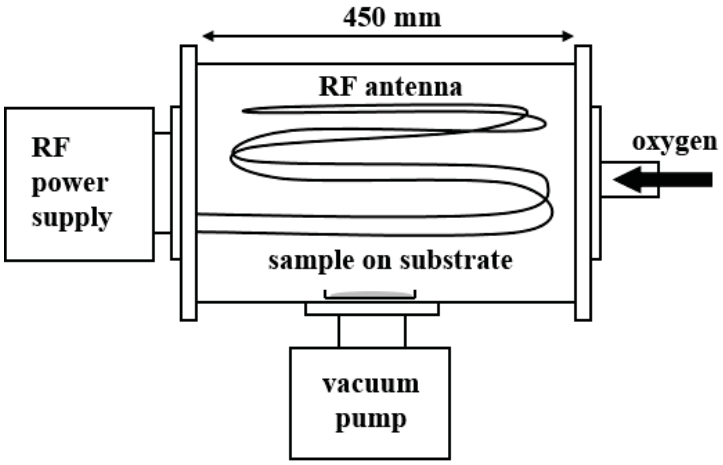

FIG. 1: Schematic of RF plasma device

were utilized for growth observation and gene analysis, respectively. After the plasma irradiation, 50 dried seeds of *Arabidopsis* were ground up and 1 μg RNA was extracted using the RNA extraction reagent. The quality of the extracted RNA was confirmed by electrophoresis. Gene expression of seeds was analyzed by the microarray method using a microarray scanner (Agilent SurePrint G3 GE 8x60K v2). The obtained gene data were arranged using the functional annotation bioinformatics microarray method and pathway analysis method using the database for annotation, visualization, and integrated discovery (DAVID).[20,21] The microarray analyses of same condition were repeated twice, and repeatability of the result was confirmed. The antioxidative activity was estimated as amount of thiol compounds in plants, which was measured using the thiol quantification reagent (ANASPEC, SensoLyteR Thiol Quantitation Assay Kit). The weight of each plant sample was 0.17 g and was kept constant throughout the experiments.

III. RESULTS AND DISCUSSION

A. Active Oxygen Irradiation to Seeds

Active oxygen species generated in the oxygen plasma by the RF discharge at low pressure were measured using optical emission spectroscopy. In the lower pressure region around 20 Pa, atomic oxygen O(^{5}P) and oxygen ions tend to be generated. When the pressure increases to 60 Pa, excited oxygen molecules such as O_2 ($^1\Sigma_g^+$) are produced due to the lower-energy electrons.[9,10] The lifetime of an excited oxygen molecule is on the order of milliseconds in low-pressure conditions. Therefore, the pressure in the chamber was varied within a range of several tens of Pa. Also, the chemical indicator shows generation of active oxygen species in the afterglow region of the chamber.

In this experiment, seeds of the radish sprout and the *Arabidopsis thaliana* were utilized for the growth observation and the gene analysis, respectively. Germination of radish sprouts was observed after 1 and 2 days from planting. The germination rate increased significantly after plasma irradiation of seeds.[9] When seeds that were irradiated by active oxygen species under particular conditions grew, stem and root lengths were longer than those of seeds without irradiation.[10] Figure 2 shows tendencies of the light emission intensity of excited oxygen molecule O_2 ($^1\Sigma_g^+$), antioxidative activity, and the full length of radish sprout varying gas pressure. Student *t*-tests were performed between lengths of untreated and plasma irradiated radish sprouts. P-values of all data used in this manuscript were < 0.05. The population served for the student *t*-test was 30.

Each curve in Fig. 2 has a peak at the same pressure, 40 Pa. An inverse relationship was observed between the intensity of O_2 ($^1\Sigma_g^+$) and the full length of radish sprout as the oxygen gas pressure changed.[10] At 40 Pa pressure, the growth of plants was suppressed by the oxidative stress due to the excess irradiation by active oxygen species. The suppression of growth factor led to expression of antioxidative genes, and large quantity of antioxidative substances was produced. On the other hand, irradiation of an appropriate amount of active oxygen species at < 20 Pa or > 60 Pa oxidized thiol compounds related to biological reactions in cells. Figure 2 also shows the relationship between

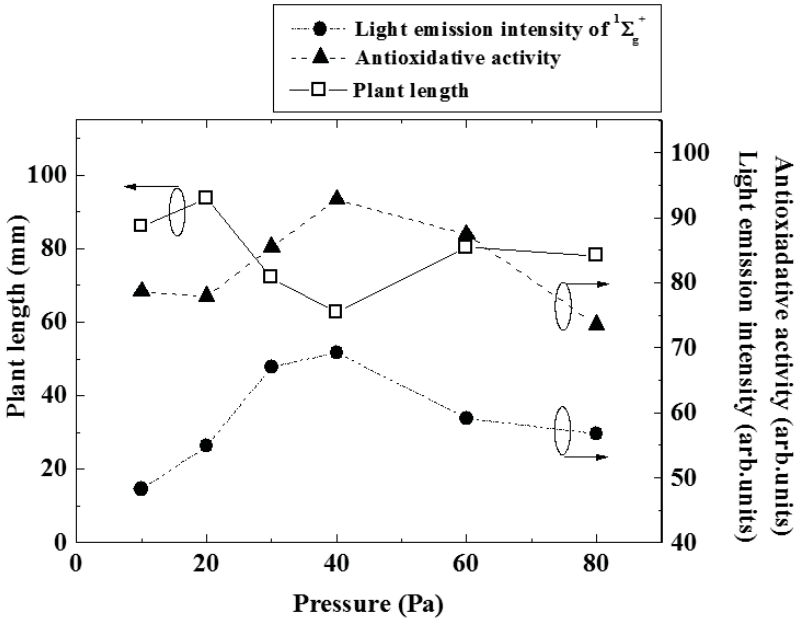

FIG. 2: Tendencies of light emission intensity of O2 (1Σ+ g), antioxidative activity, and full length of radish sprout varying gas pressure

the intensity of O_2 ($^1\Sigma_g^+$) and the amount of antioxidative substances in plant cells when varying the oxygen gas pressure. Antioxidative substances were produced depending on the oxygen plasma dose to the seeds. This finding supports the above result for O_2 ($^1\Sigma_g^+$) irradiation and plant growth. The increase of antioxidative substances, i.e., reduction-type thiol compounds, provided a counter-reaction against oxidative stress. Microscopic observation of plants indicated that the sizes of cells in stems and leaves grown from plasma-irradiated seeds were almost the same as those without plasma irradiation. These results imply that a number of cell processes increase due to excited oxygen molecules, and therefore the cell cycle is accelerated.

B. Gene Analysis of Seeds Irradiated by Oxygen Plasma

Excited oxygen molecules will penetrate the inner part of a seed through the seed coat when seeds are irradiated by oxygen plasma. However, some counter-reactions against oxidation are expected to occur. To investigate the functions of the expressed genes of seeds that were induced by oxygen plasma irradiation, gene expression analyses were performed using the functional annotation bioinformatics microarray method. Figure 3

shows a functional annotation chart of the biological processes obtained by gene ontology analysis, which was derived from seeds just after being irradiated by oxygen plasma. Functional annotations were obtained by functional analysis of genes whose expression levels were 4 times higher than the original (standard) level. The annotation chart in Fig. 3 indicates that functions of expressed genes are categorized into cell growth, stress response, photosynthesis, hormone response and others. Results of the microarray scanning of RNAs of *Arabidopsis* seeds and the gene ontology analysis indicated that the expression of 678 genes increased after oxygen plasma irradiation. When *Arabidopsis* seeds were irradiated by oxygen plasma, major functional gene categories related to cell growth or cellular metabolism, such as photosynthesis, carbon fixation, glycolysis, and the citrate cycle (TCA cycle)[25,26] showed increased expression by irradiation of active oxygen species.

When seeds were irradiated by active oxygen species, genes coding enzymes such as ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO),[27] which catalyzes the ratecontrolling reaction of photosynthesis and carbon fixation, were expressed. Active oxygen species in oxygen plasma should oxidize thioredoxin system on enzymes causing thiol bases to form disulfide bonds, thus enhancing their activities. This hypothesis is supported by the results shown in Fig. 2. Carbon fixation processes such as photosynthesis and the Calvin-Benson cycle[28] were then activated. Figures 4(a) and (b) illustrate the signal pathway diagram of the carbon fixation process and the TCA cycle, respectively, which were determined using the microarray analysis. Upregulation of some reactions involved in starch production by the Calvin-Benson cycle led to an increase in pyruvic acid generation through glycolysis, as shown in Fig. 4(a). Also, in the signal pathway diagram of the TCA cycle illustrated in Fig. 4(b), the downstream reactions of productions of oxaloacetate, marate, fructose, and bisphosphorate were upregulated by the plasma irradiation of seeds, leading to the enhancement of the TCA cycle. Therefore, the production process of the adenosine triphosphate (ATP) in plants was accelerated by oxygen plasma irradiation. Finally, an increase in ATP enhanced the cell cycle, the number of cells increased; consequently, plant growth was enhanced. Results of the GO analysis indicate gene expressions of cell cycle (GO:0007049) and cell division (GO:0051301). Due to the upregulation of the gene expression related to ATP production, materials for cell production became enriched and cell division was enhanced. Also, gene expressions of cell growth (GO:0016049), regulation of cell size (GO:0008361), unidimensional cell growth (GO:0009826), and cell morphogenesis (GO:0000902) were upregulated. These findings suggest that the size of cells increased in addition to cell division enhancement.

To determine changes in gene expressions caused by active oxygen irradiation, heat maps were constructed from the functional annotation chart obtained by gene ontology analysis. The heat maps shown in Fig. 5 were obtained using genes concerning (a) carbon fixation in photosynthetic organisms and (b) the TCA cycle. In both Fig. 5(a) and (b), upregulation was significant in genes when seeds were irradiated by active oxygen species. In the carbon fixation processes in photosynthetic organs, genes for RubisCO, GAP, and malate dehydrogenase (MDH) were upregulated, and genes for

cell growth
GO:0042547 cell wall modification during multidimensional cell growth
GO:0009828 plant-type cell wall loosening
GO:0007047 cell wall organization
GO:0042545 cell wall modification
GO:0009827 plant-type cell wall modification
GO:0009825 multidimensional cell growth
GO:0009831 plant-type cell wall modification during cell growth
GO:0009826 unidimensional cell growth
GO:0008361 regulation of cell size
GO:0032535 regulation of cellular component size
GO:0043094 cellular metabolic compound salvage
GO:0010033 response to organic substance
GO:0019253 reductive pentose-phosphate cycle
GO:0000902 cell morphogenesis
GO:0032989 cellular component morphogenesis
GO:0040007 growth
GO:0016049 cell growth
GO:0009664 plant-type cell wall organization
stress response
GO:0009628 response to abiotic stimulus
GO:0006970 response to osmotic stress
GO:0009266 response to temperature stimulus
GO:0009409 response to cold
GO:0009651 response to salt stress
photosynthesis
GO:0009637 response to blue light
GO:0010114 response to red light
GO:0009853 photorespiration
GO:0009639 response to red or far red light
GO:0019685 photosynthesis, dark reaction
GO:0009416 response to light stimulus
GO:0015979 photosynthesis
GO:0019252 starch biosynthetic process
GO:0009314 response to radiation
GO:0015977 carbon utilization by fixation of carbon dioxide
hormone response
GO:0045229 external encapsulating structure organization
GO:0048589 developmental growth
GO:0060560 developmental growth involved in morphogenesis
GO:0009725 response to hormone stimulus
others
GO:0009415 response to water
GO:0051258 protein polymerization
GO:0006949 syncytium formation
GO:0009719 response to endogenous stimulus

FIG. 3: Major functions of expressed genes of *Arabidopsis* seeds irradiated by oxygen plasma

phytoene desaturase (At4G) were downregulated as a result of the plasma irradiation. The RubisCO and GAP genes worked in the initial stage of the carbon fixation of plants; therefore, the energy production processes in photosynthesis (Fig. 4a) were enhanced by plasma irradiation. In the citrate cycle, genes for MDH and ATP citrate lyase (ACLA), which catalyze the reactions in the citrate cycle and enhance the generation of ATP, were

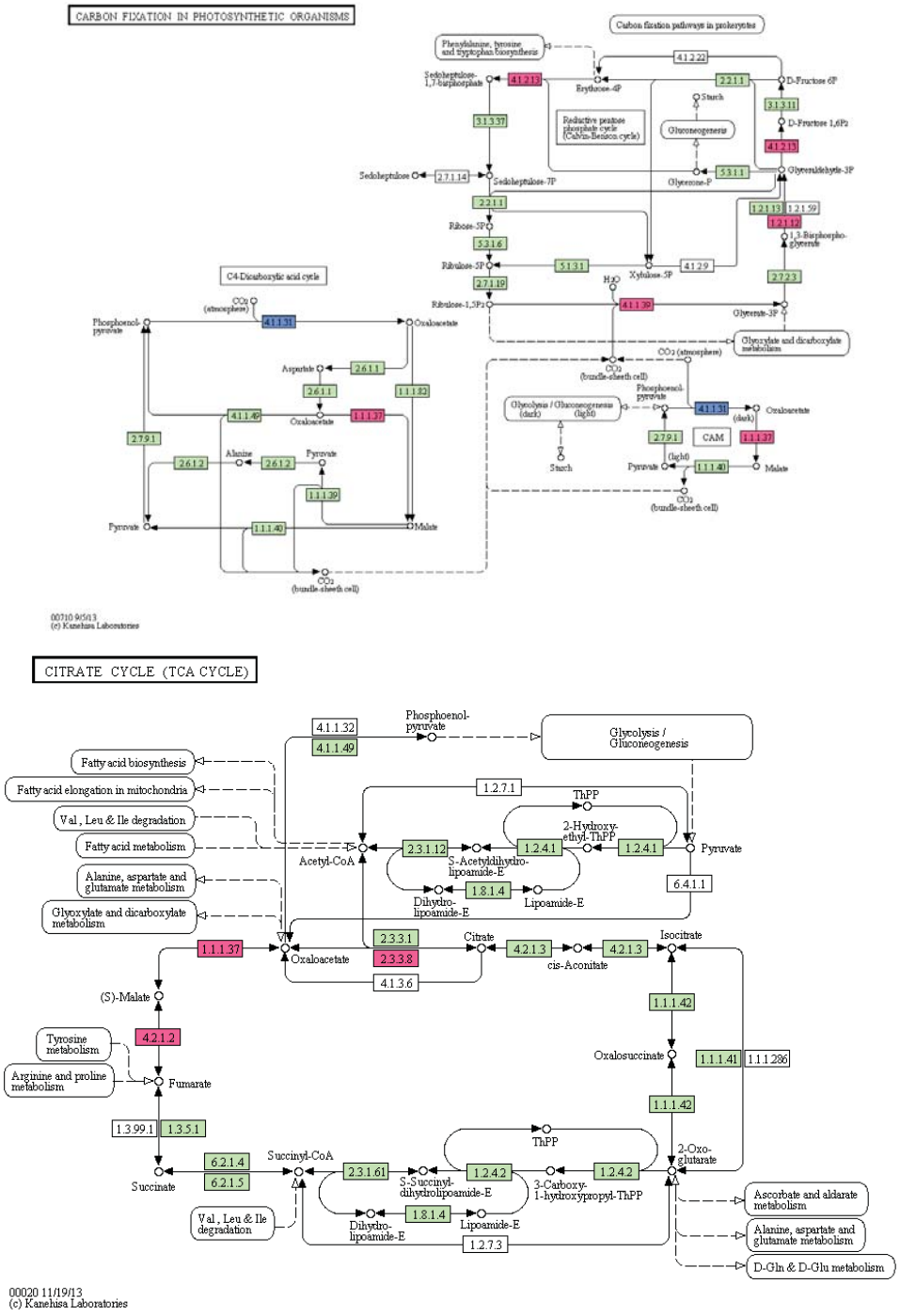

FIG. 4: (a) Schematic diagram of photosynthesis indicating upregulated reactions. (b) Schematic diagram of TCA circuit indicating upregulated reactions

upregulated by the plasma irradiation to seeds. In addition, genes of the mitochondrial lipoamide dehydrogenase and fumonisin were downregulated. These gene expressions indicate that some reactions in the TCA cycle were enhanced by oxygen plasma irradiation. Genes such as *AT5G43330* and *PMD2*, which are oxidoreductases of enzymes working in metabolic processes of plant, are commonly upregulated in TCA cycle and carbon fixation processes.

Functional annotation charts and heat maps also indicated that genes related to auxin hormone generation were upregulated, as well as those involved in photosynthesis and carbon fixation. Auxins[29–32] are a class of plant hormone that influence plant growth significantly, even in small amounts. Increases in auxin in plants leads to enhanced enzyme production and an increase in the cell longitudinal length; therefore, the length of the plant is expected to increase. Although the optimal auxin amount has not been quantified, the genes concerning auxin production were upregulated by oxygen plasma irradiation. Therefore, the enhancement of auxin production is a factor of plant growth enhancement.

Our gene ontology analysis and pathway analysis indicated that some genes concerning cell elongation proteins were activated when seeds were irradiated by active oxygen species. From the experimental results of plant cultivation after plasma irradiation, the lengths of the leaves and stems of *Arabidopsis* increased approximately 1.5 times over the control. Also, the area of leaves increased two times over the control. On the other hand, the diameter of the stems did not change. These findings indicate that the production of plant hormones was enhanced by an increase in the activity of transcription factors that regulate genes concerning hormone production such as L-tryptophan pyruvate aminotransferase (TAA1).

Our results illustrate the growth enhancement of plants due to (1) enhancement of energy production of plants via photosynthesis and carbon fixation, and (2) production of auxins in seeds. Figure 6 summarizes the sequence of reactions in seeds via reactions (i). The photosynthesis process was enhanced by active oxygen species in plasma due to activation of enzymes such as RuBisCO. Activation of photosynthesis led to enhancement of the downstream reactions sequentially.

C. Gene Analysis of Second Generation Seeds

Growth enhancement of plants grown from second-generation seeds can be used to confirm the inheritance of the growth enhancement effect. Second-generation seeds were collected from plants that grew from seeds that had undergone plasma irradiation (i.e., first generation). The growth of second-generation plants was almost the same as for seeds without plasma irradiation. This result implies that no mutaion occurred on the genes related to the growth enhancement. To determine gene expressions of second-generation seeds, gene functional annotation analysis of *Arabidopsis* second-generation seeds was performed. Figure 7 shows the functional annotation chart of the seeds. The gene ontology analysis shows that the gene expression of the second-generation seeds was different from that of first-generation seeds irradiated by oxygen plasma. There

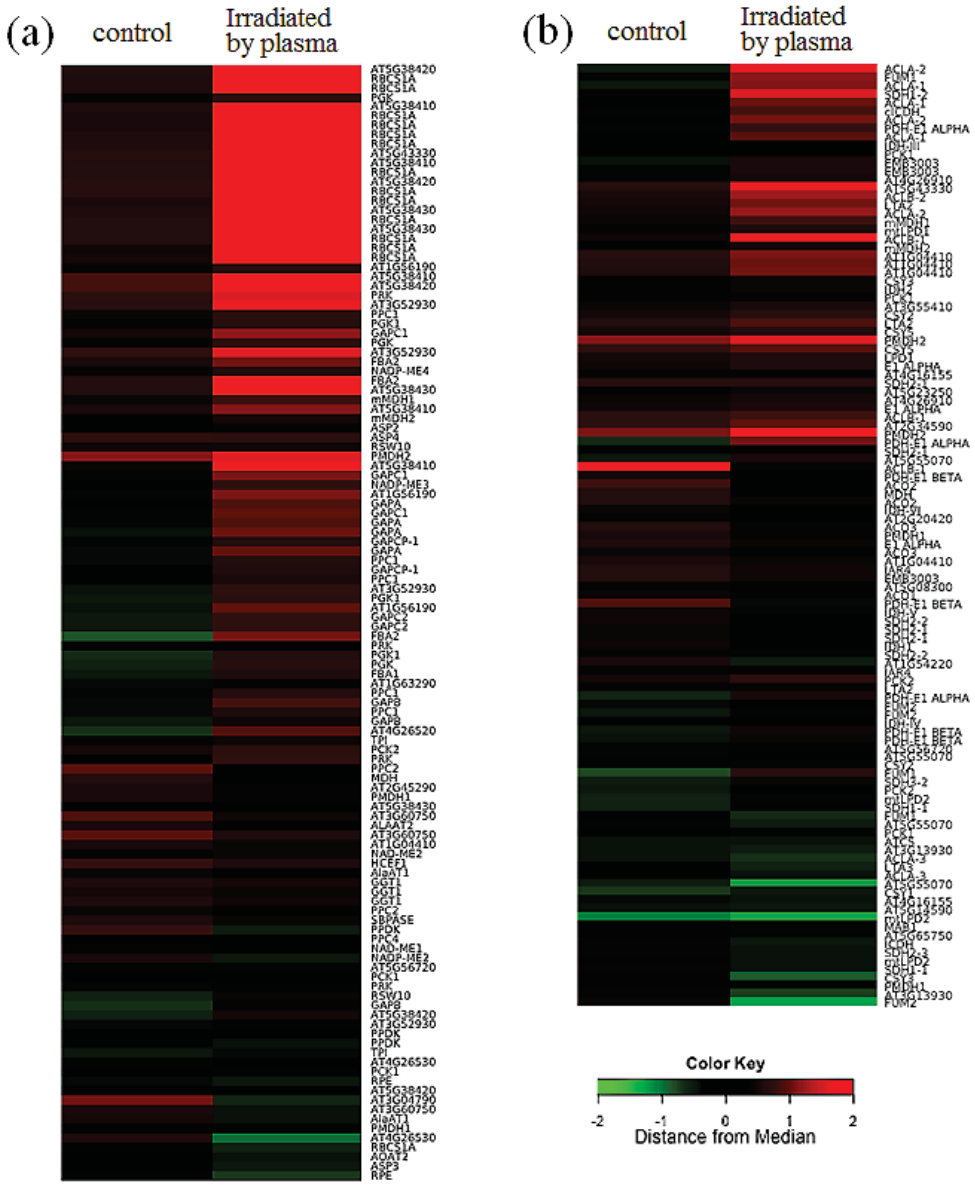

FIG. 5: Heatmaps of photosynthesis (a) and TCA circuit (b) obtained from each expressed genes

were only 50 genes with genetic variations, which is common when comparing the first generation of seeds irradiated by oxygen plasma with the second generation. In second-generation seeds, the response to oxidative stress and the secondary metabolism of plants was significant among the expressed genes, including the induction of toxic

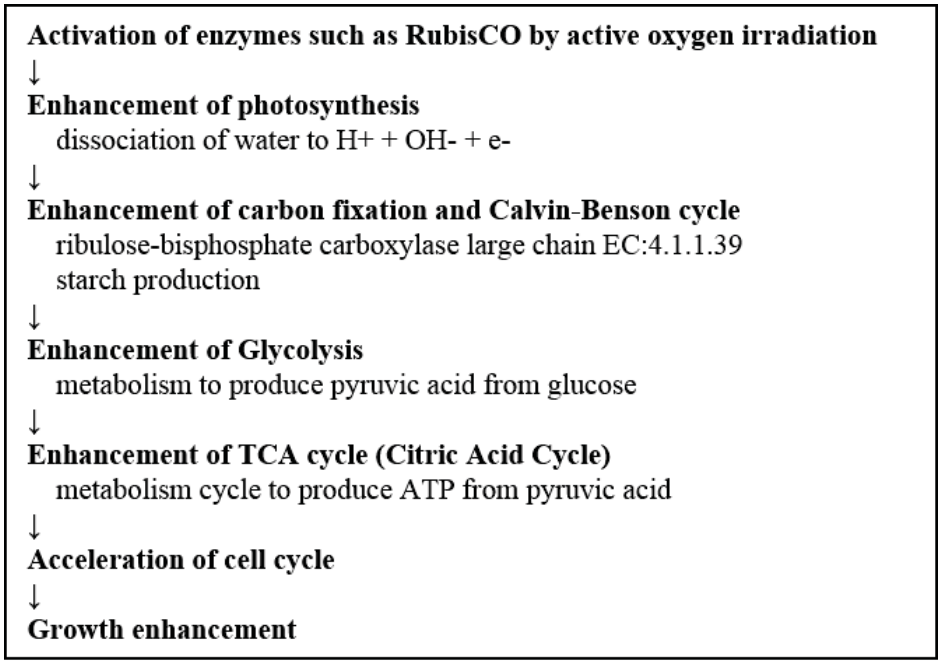

FIG. 6: Theorized pathway sequence from plasma irradiation to plant growth enhancement

catabolic processes and phenylpropanoid metabolism. Phenylpropanoids, having a hydroxyl base belonging to polyphenol moieties, indicates reducibility. The expression of genes concerning phenylpropanoids is a reaction against active oxygen irradiation. On the other hand, genes of photosynthesis, carbon fixation, and the TCA cycle did not show differential expression, which was prominent in the first-generation seeds. These results imply that the second-generation plant obtains a resistivity against the active oxygen species. Also, gene expression related to plant growth in the second-generation seeds was similar to that of the first generation.

These results indicate that the gene expression in seeds of the second generation was significantly different from that of the first generation irradiated by plasma. The enhancement of cell growth and cellular metabolism such as photosynthesis and carbon fixation, which were upregulated in the first-generation seeds by oxygen plasma irradiation, was not passed on to the next generation. Because the chemical energy of active oxygen species produced in this experiment was approximately 4 eV, active oxygen species hardly modified DNA sequences and mutations were not expected. These results indicate that DNA related to energy production was not modified by active oxygen irradiation. Therefore, growth enhancement was not observed in plants grown from second-generation seeds. The active oxygen species enhanced some transcription processes in the first generation due to oxidation of enzymes involved in energy production; genes concerning energy production were then activated. Therefore, the growth enhancement

GO	Count	PValue
GO:0019748~secondary metabolic process	19	4.11E-09
GO:0019439~aromatic compound catabolic process	7	6.36E-08
GO:0042545~cell wall modification	11	9.44E-08
GO:0052482~cell wall thickening during defense response	5	5.89E-06
GO:0052544~callose deposition in cell wall during defense response	5	5.89E-06
GO:0010200~response to chitin	9	6.49E-06
GO:0052542~callose deposition during defense response	5	9.79E-06
GO:0052543~callose deposition in cell wall	5	9.79E-06
GO:0052386~cell wall thickening	5	1.23E-05
GO:0052545~callose localization	5	1.53E-05
GO:0042343~indole glucosinolate metabolic process	4	1.57E-05
GO:0042434~indole derivative metabolic process	6	1.77E-05
GO:0042430~indole and derivative metabolic process	6	1.77E-05
GO:0033037~polysaccharide localization	5	1.88E-05
GO:0009743~response to carbohydrate stimulus	10	2.48E-05
GO:0006952~defense response	22	3.88E-05
GO:0010033~response to organic substance	22	2.54E-04
GO:0016143~S-glycoside metabolic process	5	3.72E-04
GO:0019760~glucosinolate metabolic process	5	3.72E-04
GO:0019757~glycosinolate metabolic process	5	3.72E-04
GO:0006790~sulfur metabolic process	8	3.76E-04
GO:0042436~indole derivative catabolic process	3	5.92E-04
GO:0009617~response to bacterium	9	6.35E-04
GO:0055114~oxidation reduction	21	7.57E-04
GO:0009723~response to ethylene stimulus	9	8.69E-04
GO:0007047~cell wall organization	9	0.001439
GO:0042219~cellular amino acid derivative catabolic process	4	0.001448
GO:0019438~aromatic compound biosynthetic process	8	0.00145
GO:0009808~lignin metabolic process	5	0.001635
GO:0009725~response to hormone stimulus	17	0.001749
GO:0033554~cellular response to stress	11	0.001785
GO:0009698~phenylpropanoid metabolic process	7	0.001881
GO:0006955~immune response	9	0.002048
GO:0045229~external encapsulating structure organization	9	0.002048
GO:0042435~indole derivative biosynthetic process	4	0.002944
GO:0042742~defense response to bacterium	7	0.003147
GO:0010035~response to inorganic substance	12	0.003208
GO:0009719~response to endogenous stimulus	17	0.00353
GO:0016137~glycoside metabolic process	5	0.004163
GO:0009407~toxin catabolic process	4	0.005467
GO:0009404~toxin metabolic process	4	0.005467
GO:0045087~innate immune response	8	0.005683
GO:0006575~cellular amino acid derivative metabolic process	8	0.006256

FIG. 7: Major functions of expressed genes of second generation seeds of *Arabidopsis*

phenomena observed in the first generation owing to enhancement of gene expressions is likely epigenetic. The epigenetics that change gene expression are not necessarily related to genetic mutations. Because the growth enhancement effect of plants observed in this study was not inherited by the next generation, a genetic mutation was not induced by the oxygen plasma irradiation. On the other hand, the growth enhancement effect was preserved during cell divisions in the first generation. Therefore, the observed gene expression is related to epigenetics.

IV. CONCLUSION

The excited oxygen molecules produced by oxygen plasma induce gene expression of photosynthesis and carbon-fixation processes concerned with cell growth or cellular metabolism and plant hormone generation. Disappearance of the growth enhancement effect in the second generation indicates that a genetic mutation in the seed DNA was not induced by oxygen plasma irradiation; therefore, the epigenetic effect in the first generation was a mechanism of plant growth enhancement.

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