Temperature-controlled atmospheric-pressure plasma treatment induces protein uptake via clathrin-mediated endocytosis in tobacco cells

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Received November 16, 2021; accepted January 5, 2022 (Edited by K. Mishiba)

Abstract
Previously, we developed a method that uses temperature-controlled atmospheric-pressure plasma to induce protein uptake in plant cells. In the present work, we examined the mechanism underlying such uptake of a fluorescent-tagged protein in tobacco leaf cells. Intact leaf tissue was irradiated with N2 plasma generated by a multi-gas plasma jet and then exposed to the test protein (histidine-tagged superfolder green fluorescence protein fused to adenylate cyclase); fluorescence intensity was then monitored over time as an index of protein uptake. Confocal microscopy revealed that protein uptake potential was retained in the leaf tissue for at least 3 h after plasma treatment. Further examination indicated that the introduced protein reached a similar amount to that after overnight incubation at approximately 5 h after irradiation. Inhibitor experiments revealed that protein uptake was significantly suppressed compared with negative controls by pretreatment with sodium azide (inhibitor of adenosine triphosphate hydrolysis) or sucrose or brefeldin A (inhibitors of clathrin-mediated endocytosis) but not by pretreatment with genistein (inhibitor of caveolae/raft-mediated endocytosis) or cytochalasin D (inhibitor of micropinocytosis/phagocytosis), indicating that the N2 plasma treatment induced protein transportation across the plant plasma membrane via clathrin-mediated endocytosis.

Key words: atmospheric-pressure plasma, clathrin-mediated endocytosis, plasmaized gas, protein introduction, reactive species.

Techniques for introducing proteins into plant cells are useful in plant research, particularly in the domains of genome editing and protein functional analysis. Previously, we developed a novel means of introducing proteins into plant cells that used temperature-controlled atmospheric-pressure plasma, although at that time the underlying mechanism was unclear (Yanagawa et al. 2017). Recently, in mammalian cells, it was reported that irradiation of medium containing MCF-7 breast cancer cells with atmospheric-pressure argon plasma induced uptake of ribonucleoprotein into the cells via both clathrin-mediated and caveolae-mediated endocytosis (Cui et al. 2021). It has also been reported that uptake of plasmid DNA via clathrin-mediated endocytosis is induced in mouse fibroblast L929 cells by treatment of the cells with atmospheric-pressure air plasma (Jinno et al. 2017). However, there are several important differences between our approach and these other reported approaches. A major difference is that in our approach intact plant tissues such as leaves and roots are directly irradiated with the plasma (Yanagawa et al. 2017). Another difference is that our plasma was generated by applying high voltage to pre-cooled N2 or CO2 gas only after the glow discharge containing the reactive species was pushed out from the small outlet of the grounded plasma-generating apparatus, which ensured that no electric stimulus was applied to the experimental samples (Figure 1). In the approaches using mammalian cells, the cells were exposed not only to a chemical stimulus but also to an electric stimulus. For example, in the system of Jinno et al. (2017), which uses dielectric barrier discharge, the cells are exposed to an electrical stimulus because they are placed in the area where the discharge is generated by applying a high voltage. Similarly, in the system of Cui et al. (2021), argon plasma was generated by using a plasma jet that
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is similar shape to our plasma generating apparatus; however, the non-temperature-controlled plasma emitted from the outlet includes not only reactive species but also an electric stimulus. The types and amounts of reactive species in plasma vary depending on the generation method, gas species, and temperature (Kawano et al. 2018; Takamatsu et al. 2014); therefore, the differences between techniques may affect the mechanism by which macromolecules are taken up into cells.

Here, we examined the mechanism underlying N₂ plasma-induced uptake of a fluorescent-tagged protein (histidine-tagged superfolder green fluorescence protein fused to adenylate cyclase; His-sGFP-CyaA) into the cells of intact leaf pieces of tobacco plant (Nicotiana tabacum cv. Samsun NN). Previously, we reported that temperature control was necessary for introducing proteins into the cells of intact plant tissues (Yanagawa et al. 2017); therefore, here we used a multi-gas plasma jet and a refrigerant cooled by a chiller (EYELA NCB-1210) to produce temperature-controlled N₂ plasma. The temperature of the N₂ plasma was kept within the 20°C range (See Supplementary Figure S1).

First, we examined the amount of time after plasma irradiation within which protein can be introduced into the plant cells (Figure 2). Pieces of tobacco leaf were irradiated with plasma, soaked in distilled water for various amounts of time, and then soaked in phosphate-buffered saline (PBS) containing 50 µg ml⁻¹ of the test protein (histidine-tagged superfolder green fluorescence protein fused to adenylate cyclase), which was prepared for a previous study (Yanagawa et al. 2017). As a positive control, leaf pieces were treated with plasma irradiation and then placed directly in the protein solution (Direct). As a negative control, leaf pieces not treated with plasma irradiation were placed directly in the protein solution (No treatment). After soaking overnight, the leaf pieces were washed once with 1×PBS and fluorescence was observed under a confocal microscope (B). (C) Relative fluorescence intensity was calculated in the Image J software ver. 1.80 as the average of three of the brightest areas (each 100 px×100 px square) observed under the confocal microscope. Note that the intensity of first measured sample in “Direct” was set to 1.0. Bars, 200 µm.

intensity was comparable with that in the leaf pieces not exposed to the irradiation, indicating that the plasma-induced protein uptake persisted for at least 3 h.
Next, we examined the time course of protein uptake by the cells (Figure 3). Plasma-treated leaf pieces were soaked first in a solution containing His-sGFP-CyaA for various lengths of time and then in distilled water overnight. After plasma treatment followed by exposure to the protein for 10 s, the fluorescence intensity was comparable to that observed without plasma treatment. However, the fluorescence intensity began to increase from a soaking time of 30 s until the intensity after soaking for 5 h which was comparable to that observed after soaking overnight, indicating that protein uptake saturated at approximately 5 h after irradiation.

The continued uptake of protein suggested involvement of an active transport process such as endocytosis. To verify this hypothesis, we exposed plasma-treated irradiated leaf pieces to one of several endocytosis inhibitors prior to exposure to His-sGFP-CyaA (Figure 4). As negative controls, plasma-treated leaf pieces were soaked in PBS buffer containing no solvent (for sodium azide and sucrose) or each solvent that is ethanol (for genistein) or methanol (for brefeldin A and cytochalasin D) prior to exposure to the protein. Compared with control, the fluorescence intensity was significantly decreased in leaf pieces pretreated with sodium azide, an inhibitor of adenosine triphosphate hydrolysis, which is essential for the production of the energy used in the endocytosis process. Pretreatment with sucrose or brefeldin A, which are inhibitors of clathrin-mediated endocytosis, also resulted in significantly reduced fluorescence intensity compared with control. In contrast, no significant changes in fluorescence intensity were observed in the leaf pieces pretreated with genistein, an inhibitor of caveolae/raft-mediated endocytosis, or with cytochalasin D, an inhibitor of micropinocytosis/phagocytosis compared with control. Previous reports showed that both genistein and cytochalasin D inhibited the functions of their experimental targets in plants (Arase et al. 2012; Tiwari and Polito 1990), indicating that they should be worked in the tobacco leaves. Therefore, these findings indicate that exposure to N₂ plasma induced clathrin-mediated endocytosis in the plasma membrane of the plant cells.

The present data indicated that protein uptake across the plasma membrane of tobacco leaf cells after treatment with N₂ plasma was via clathrin-mediated endocytosis. Our present finding is inconsistent with a recent report that both clathrin-mediated and caveolae-mediated endocytosis of macromolecules are induced by plasma irradiation in mammalian cells (Cui et al. 2021). Our apparatus applies plasmaized gas containing reactive species, but not electric stimuli, to the experimental samples, indicating that the observed clathrin-mediated endocytosis was activated only by the reactive species in the plasma and not by an electric stimulus. In our previous study, we found that N₂ and CO₂ plasmas were more effective than Ar, O₂, air, or H₂/Ar plasmas for introducing proteins into plant cells (Yanagawa et al. 2017), suggesting that reactive oxygen or nitrogen species generated in the plasma jet are important for plasma-mediated protein uptake in plant tissues. In this study, we used N₂ plasma, which contains more hydroxyl radical compared with the amount in other gas plasmas (Takamatsu et al. 2014). This suggests that hydroxyl radical might play a major role in inducing protein uptake across the plasma membrane of tobacco

![Figure 3](image-url)

**Figure 3.** Time course analysis of protein entry into cells. (A) Schematic overview of the treatment process. Each tobacco leaf piece was irradiated with N₂ plasma for 5 s; soaked in 1×PBS containing 50 µg ml⁻¹ of the test protein for 10 s, 30 s, 1 min, 5 min, 30 min, 1 h, 2 h, 3 h, or 5 h; and then soaked overnight at 22°C in water. As a positive control, leaf pieces were treated with plasma irradiation and then soaked overnight in the protein solution (O/N). As a negative control, leaf pieces not treated with plasma irradiation were placed directly in the protein solution for 10 s before being transferred to the water (No plasma). After soaking overnight, the leaf pieces were washed once with 1×PBS and fluorescence was observed under a confocal microscope (B). (C) Relative fluorescence intensity was calculated in the Image J software ver. 1.8.0 as the average of three of the brightest areas (each 100 px × 100 px square) observed under the confocal microscope. Note that the intensity of first measured sample in “O/N” was set to 1.0. Bars, 200 µm.
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leaf cells by clathrin-mediated endocytosis. Our next task will be to clarify the types and amounts of reactive species involved in the observed protein uptake. Plant cells have cell walls unlike to mammalian cells. Since the cell walls are permeable of molecules with a diameter of at least 5 nm (Fisher 2000), they cannot be a barrier of protein introduction. Intact plant leaves have special structure such as cuticular and wax layers at their surface. It is known that atmospheric-pressure plasma can hydrophilize the surface of treated samples (Takamatsu et al. 2013), implying that the hydrophilization allowed the protein to pass through their structures. It will be elucidated in the future.

Acknowledgements

We thank Y. Suzuki and S. Gonokami for their technical assistance. This work was partly supported by the research program on development of innovative technology grants from the Project of the Bio-oriented Technology Research Advancement Institution (BRAIN) and Cabinet Office, Government of Japan, Cross-ministerial Strategic Innovation Promotion Program (SIP), “Technologies for smart bio-industry and agriculture” (funding agency: Bio-oriented Technology Research Advancement Institution, NARO) to IM. Part of this research was supported by the Cooperative Research Project of the Research Center for Biomedical Engineering (to YY and IM).

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