Selective observation of starch in a water plant using optical sum frequency microscopy

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Abstract

The photosynthesis, transfer, and storage of starch are the most important biogenic processes occurring in plants. In order to observe the colorless and transparent starch granules in a plant, a chemical pretreatment such as staining of the starch is currently required, which seriously damages the tissue cells in the plant. In this study, it is demonstrated that non-destructive chemical analysis of starch granules in a plant can be performed by using optical second harmonic and sum frequency microscopy. This novel technique for *in vivo* analysis will provide epoch-making information about saccharides in a plant and can be extended to the analysis of many other materials, from living tissue to semiconductors.

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Introduction

The photosynthesis of starch, and its transfer and storage, are the most important biogenic processes occurring in a living plant. Observation of the colorless and transparent starch granules in a plant with a conventional optical microscope requires chemical pretreatment, i.e., removing the chlorophyll and then staining the starch with an iodine aqueous solution. However, since this treatment seriously damages the tissue cells, it cannot be used for an *in vivo* analysis of starch in a living plant. Recently, we found that starch granules have large second-order optical nonlinearity and can be observed selectively in a plant without any pretreatment, using an optical second harmonic (SH) microscope [1,2]. However, it was also found that the SH microscope could not identify constituent saccharides in the starch. Here we show that molecular vibrational images of starch granules can be obtained for chemical analysis by detecting sum frequency (SF) light from a plant specimen irradiated with visible and tunable IR light beams.

Optical second harmonic generation (SHG) and optical sum frequency generation (SFG) are the lowest-order nonlinear optical processes that occur in a medium without inversion symmetry [3]. Biological materials consist of various kinds of chiral molecules. Their structures lack inversion symmetry at the molecular level, and they can show second-order optical nonlinearity.

If asymmetric molecular units are well oriented, so that their higher-order structure has macroscopic asymmetry, strong SH or SF light can be generated by the resonant excitation of the electronic or vibrational energy levels of the molecules. In particular, molecular species can be distinguished in the vibrational images from SF microscopy [4]. However, only a small number of studies have identified organic or inorganic materials [5,6] and no studies of living organisms have been done using SF microscopy. In this study, a water plant, *Chara fibrosa* (Fig. 1a), was chosen as the subject and the identification of chiral molecules in it was attempted. The internodal cells forming the stems of *Chara fibrosa* are large enough to be clearly observed with a conventional optical microscope, and they have long been used for experiments on the electrical properties of living plant cells [7]. Since the starch granules stored as nutrition in *Chara fibrosa* consist of noncetrosymmetric and optically active D-glucopyranose units, a large nonlinear optical response from them was expected.

Experimental

The setups of the SH and SF microscopes have been described in detail in our previous papers [1,2,4,8]. Briefly, the excitation light for SH intensity image measurements was generated by a mode-locked Nd^{3+}:YAG laser with a wavelength of 1064 nm, a pulse duration of 30 ps, and a repetition rate of 10Hz. The pulse energy of the incident light at the sample surface was

~100μJ/pulse. In the SF intensity image measurements, we used visible light at wavelength 532nm as the doubled frequency output from the mode-locked Nd^{3+}:YAG laser, and wavelength tunable infrared light as output from an optical parametric generator, with an amplifier system driven by the fundamental and SH output of the same Nd:YAG laser. The spectral bandwidth of IR light was $6cm^{-1}$. The pulse energies of the visible and IR beams at the sample surface were ~2μJ/pulse and ~40μJ/pulse, respectively. The polarizations of the two beams were parallel to each other. The scattered SH and SF light was passed through an objective lens and a band-pass filter and detected by a charge-coupled device camera with a time-gated image intensifier (II-CCD). The center wavelengths of the band-pass filters for the SH and SF measurements were 532nm and 460nm, respectively. In the SH intensity image measurements, we confirmed that the observed signal contained no multiphoton-induced luminescence light from plant samples. In the SF intensity image measurements, when the IR light irradiation was blocked, weak signals were observed due to the multiphoton-induced luminescence excited by the incident visible light. This contribution from the luminescence was removed by a subtraction image processing technique. For the measurement of SF intensity spectra, the sensitivity of the detection system was calibrated using an SF signal from a ZnS sintered compact.

Chara fibrosa, the plant sample for the SH and SF image observations, is a common water

plant found frequently in ponds and lakes. It was cultivated in a water tank under irradiation with a fluorescent lamp. When it was fully-grown reproductive organs, oogonia and antheridia, formed at the knots of the stems of the plant. At this stage, a part of the plant was mounted on a silicon wafer in a shallow water vessel, and was observed by SH microscopy, while a part of the plant mounted on a silicon wafer in air was observed by SF microscopy. As reference samples for the SF spectroscopy the following saccharides were used: research grade powders of D(+)-glucose (Nacalai Tesque), amylopectin from maize (Fluke), amylose (type III) from potato (Sigma), and β-cyclodextrin (Sigma Aldrich).

Results and discussion

Figures 1b and c show microphotograph and SH intensity images of living *Chara fibrosa* in water. In Fig. 1b, we can see a yellow elliptical oogonium, a red spherical antheridium, and green stems containing chlorophyll. Fig. 1c indicates that the oogonium emits strong SH light, while the other parts do not. By decoloring this sample with alcohol and staining it with iodide, it was found that the oogonium contains a large number of starch granules, with few starch granules in the other parts. Furthermore, the SH intensity images of the stained sample showed that strong SH light was emitted from every stained starch granule. Starch consists of many D-glucopyranose units linked by α-1,4-glycosidit bonds and arranged in a periodic and

well-oriented structure. Amylopectin, the main component of starch, has a higher-order structure containing branches of 1,6-glycosideitc bonds as shown in Fig. 1d [9,10]. This higher-order structure with large macroscopic asymmetry is the most probable origin of starch's high SH intensity. Cellulose, another constituent material of plants, also consists of D-glucopyranose units, but its higher-order structure is different from that of starch so that its SH intensity is weaker than that of starch by two orders of magnitude [2]. Carefully looking at Fig. 1c, we find that the SH intensity from the ellipsoid in the oogonium is not uniform; strong SH light is emitted from the edges of the oogonium. On the other hand, no distinction between the edges and the other parts of the oogonium can be seen in the linear optical image in Fig. 1b. This result demonstrates the possibility that the detailed density distribution of starch can be evaluated by analyzing the observed SH intensity image.

Starch mainly consists of two different types of polysaccharide, amylose and amylopectin. Monosaccharides such as glucose may also be contained in the plant tissue. In order to identify saccharides stored in the oogonium of *Chara fibrosa*, we used SF microscopy to observe its molecular vibrational images. Fig. 1e is a microphotograph taken in air of an oogonium of dried *Chara fibrosa*, and Figs. 1f and g are the SF intensity images of the same sample irradiated by IR light with the wavenumbers of 2905cm^{-1} and 2930 cm^{-1} in the region of the C-H stretching vibration. In the present study, dead *Chara fibrosa* was used to facilitate the experiment, but *in*

vivo observation of SF intensity images of plants in water is also possible. In Figs. 1f and g, SF light is generated only in the oogonium, which contains a large number of starch granules, and a few bright spots such as spot A are also seen. The total SF intensity at 2930cm^{-1} in Fig. 1g is considerably smaller than that at 2905cm^{-1} in Fig. 1f, while the SF intensities at spot A are similar for both wavenumbers. This result suggests that the observed SF signal is sensitive to the C-H vibrational states of the saccharide molecules in *Chara fibrosa*, and the vibrational state of spot A is different from that of the other parts of the oogonium.

We have also observed SF intensity images of this oogonium over the whole spectral range of the C-H stretching vibration (2750~3100cm^{-1}) to obtain vibrational spectra. Figs. 2a and b are the SF intensity spectra of the whole area and of spot A of the oogonium, respectively. Although these spectra are similar to each other, a slight reproducible difference in the shapes of the peaks around 2910cm^{-1} is seen. In order to obtain reference data, we also measured the SF intensity spectra of four saccharides consisting of D-glucopyranose units: amylopectin and amylose as the main components of starch, glucose as a monosaccharide, and β-cyclodextrin as an origo-saccharide, as shown in Figs. 2c-2f. The SF intensity and the shapes of the spectra depend strongly on the higher-order structures of the saccharide molecules. It is interesting to note that the SF intensity of amylose, which is known to have a double helix structure [11], is very weak. The explanation for its low SF intensity is probably that the double helixes have

macroscopic inversion symmetry or they are randomly oriented. Comparing the SF intensity spectra of the oogonium of *Chara fibrosa* in Fig. 1a and b with those of four saccharides in Fig. 1c-f, one can see that the shapes of the spectra of the oogonium resemble that of amylopectin fairly well. Thus, it seems reasonable to conclude that amylopectin is at least the dominant saccharide component of the oogonium of *Chara fibrosa*. It is also likely that the amylopectin in spot A has a slightly different higher-order structure, and its macroscopic asymmetry is large so that it emits strong SF light.

Conclusion

In this study, we have demonstrated that selective and non-destructive observation of starch granules in a water plant can be performed using nonlinear optical microscopy. The selective observation of asymmetric species in materials is the most useful advantage of SF microscopy, and it cannot be achieved by other vibrational microscopies such as Raman or IR absorption microscopy [12]. If Raman or IR microscopy is used to observe a vibrational image of C-H bonds in a plant, signals from the other hydrocarbons will overwhelm the signal from starch. Thus, nonlinear optical microscopy can be developed as a powerful tool for investigating bioprocesses such as the photosynthesis associated with starch production in a living plant. In the future this technique can be applied to many materials from living tissue to semiconductors and can be

expected to impact biology, medical science, and surface science.

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Figure legends (We'd like to print out Fig.1 as double column width)

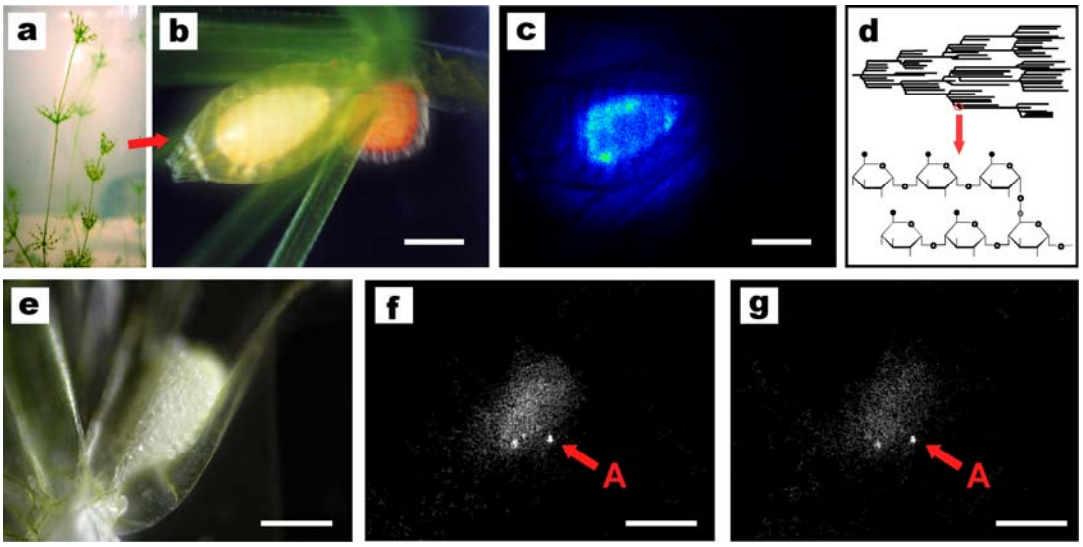

Fig. 1. Nonlinear optical images of a water plant. (a) Global photograph of *Chara fibrosa*. (b) Microphotograph and (c) SH intensity image of living *Chara fibrosa* with an oogonium (indicated by an arrow) and an antheridium in water. (d) Higher-order structure of starch consisting of D-glucopyranose units. (e) Microphotograph and (f,g) SF intensity images of dry *Chara fibrosa* with an oogonium in air. The wavenumbers of incident IR lights for the SF images are 2905cm^{-1} in (f) and 2930cm^{-1} in (g). The polarizations of the incident infrared and visible beams were parallel to each other for the SF measurement. All scale bars in the images are 200μm.

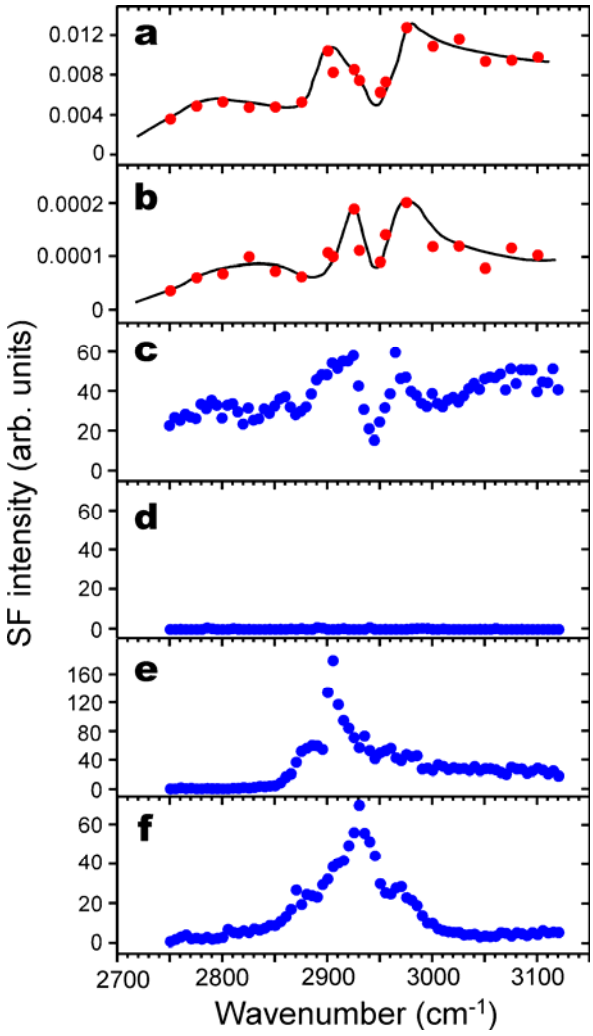

Fig. 2. SF intensity spectra of the C-H stretching vibrational mode. (a) the whole area; (b) spot A (see Figs. 1(f) and(g)) on an oogonium of *Chara fibrosa*; (c) amylopectin; (d) amylose; (e) glucose; (f) β-cyclodextrin. The polarizations of the incident infrared and visible beams were parallel to each other for the SF measurements.