Highly sensitive determination of transient generation of biophotons during hypersensitive response to cucumber mosaic virus in cowpea

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Abstract

The hypersensitive response (HR) is one mechanism of the resistance of plants to pathogen infection. It involves the generation of reactive oxygen species (ROS) which have crucial roles in signal transduction or as toxic agents leading to cell death. Often, ROS generation is accompanied by an ultraweak photon emission resulting from radical reactions that are initiated by ROS through the oxidation of living materials such as lipids, proteins, and DNA. This photon emission, referred to as ‘biophotons’, is extremely weak, but, based on the technique of photon counting imaging, a system has been developed to analyse the spatiotemporal properties of photon emission. Using this system, the dynamics of photon emission which might be associated with the oxidative burst, which promotes the HR, have been determined. Here, the transient generation of biophotons is demonstrated during the HR process in cowpea elicited by cucumber mosaic virus. The distinctive dynamics in spatiotemporal properties of biophoton emission during the HR expression on macroscopic and microscopic levels are also described. This study reveals the involvement of ROS generation in biophoton emission in the process of HR through the determination of the inhibitory effect of an antioxidant (Tiron) on biophoton emission.

Key words: Biophoton, cucumber mosaic virus, hypersensitive response, imaging, reactive oxygen species, ultraweak photon emission, visualization.

Introduction

Recently, ‘biophotons’ from plants have attracted attention for providing novel information for visualizing the physiological states of plants (Kobayashi et al., 1997; Havaux, 2003; Bennett et al., 2005; Mansfield, 2005). Imaging using biophotons offers a novel methodology for assessing plant physiology. Biophotons are spontaneous ultraweak photon emissions that originate from biochemical metabolic reactions of living organisms. Biophotons do not require external chemiluminescent or bioluminescent reagents, nor do they require transgenic techniques for labelling. In mammals too, biophoton emissions are known to occur during the immunological response; they originate from oxidative bursts in immunocytes. Similarly, the generation of biophotons in plant responses involved in resistance to infection has been revealed.

Biophoton emission, sometimes referred to as ultraweak photon emission or ultraweak/dark bioluminescence, is commonly recognized as radiation with wavelengths from visible to near-infrared induced through electronically excited molecules that exist in living cells. Emissions result from reactions involving radicals accompanying the production of reactive oxygen species (ROS). The emission intensity is $<10^{-15}$ W cm$^{-2}$, which is a level $10^{-3}$–$10^{-6}$ times lower than the visible level. Although the intensity is extremely low, photons possess information related to metabolic production of ROS associated with various types of physiological and/or pathological states in living systems. This is generally observed and

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† Professor Yoshio Ehara passed away on 21 April 2006. The authors mourn his passing.

Abbreviations: CMV, cucumber mosaic virus; HR, hypersensitive response; PCD, programmed cell death; ROI, region of interest; ROS, reactive oxygen species.
applicable for plants, animals, and microbes (Inaba, 1988; Devaraj et al., 1997; Kobayashi, 2003a, b). Various studies have analysed plant physiological responses to various stresses, including both biotic (Roscher, 1991; Makino et al., 1996; Kobayashi et al., 2003; Bennett et al., 2005; Mansfield, 2005) and abiotic stress (Kobayashi et al., 1996, 1997; Ohy et al., 2000; Havaux, 2003). A highly sensitive system has been developed for in situ imaging of biophoton emissions to visualize ROS-related physiological changes in plants, and the first image is shown of biophoton emission resulting from the hypersensitive response (HR) in the cowpea (Vigna unguiculata)–cucumber mosaic virus (CMV) system (Kobayashi et al., 2003).

The HR process is identified as a gene-for-gene theory describing the interaction of a translation product of an avirulence gene (Avr gene) of a pathogen with a ligand by a receptor encoded by a corresponding resistance gene (R gene) of a host, described as incompatible plant–pathogen interactions (Baker et al., 1997). In the case of the cowpea–CMV system, the Avr gene is known to be RNA2, which codes for the 2a protein, a subunit of viral replicase components. In addition, the R gene is identified with the Cry locus, inherited as a single dominant trait (Nasu et al., 1996; Karasawa et al., 1997, 1999). The HR results in localized cell death at the initial infection site to delimit the spread of the invading pathogen. Confinement of the virus is exhibited as the formation of local lesions with cell death within 24 h after inoculation. During the HR, ROS production, termed the oxidative burst, is known to contribute through its important role in the diverse array of defence mechanisms. As an intracellular signal for triggering the defence response and extracellular direct agents for defence, ROS mediate the expression of defence-related genes, alteration of the membrane potential, and cell wall modification through oxidative cross-linking of cell wall structural proteins and through the production of antimicrobial compounds: phytoalexins, polyphenols, and chitinase (Mehdy, 1994; Baker et al., 1997; Vranova et al., 2002). In the case of the HR in the cowpea–CMV system, ROS are thought to contribute to signal transduction, and might act as a toxic agent leading to host cell death through lipid peroxidation (Roscher, 1991). In contrast, Bennett et al. (2005) reported biophoton generation in the HR of Arabidopsis thaliana and claimed that the increases of cytosolic calcium and nitric oxide were key roles for biophoton emission, but ROS elevation was not necessary.

In this study, the transient generation of biophotons is characterized using a highly sensitive imaging technique based on a photon counting imaging system. The spatiotemporal dynamics of biophoton emissions on macroscopic and microscopic levels and the spectral distribution of biophoton emission with evaluation of ROS involvement in biophoton emissions are also described.

Materials and methods

Two-dimensional photon counting imaging and procedures for spatiotemporal analyses of biophoton emission

The imaging system comprises a two-dimensional photon counting tube that is characterized by its large active area, a highly efficient lens system installed in the sample chamber, and electronic equipment for identifying two-dimensional spatial and temporal photoelectron data (Kobayashi et al., 1996, 1999a, b). The photon counting tube (Model IPD 440; Photek, Ltd, UK) comprises a 40 mm diameter photocathode, a microchannel plate, and a resistive anode for single-photon counting imaging. The detector is incorporated into a specially designed vacuum chamber to cool the device at −35 °C for reduction of dark current and to ensure long-term stability of the device for ultimate sensitivity. This system detects wavelengths of 350–900 nm with a quantum efficiency of 15% at 500 nm, 10% at 600 nm, and 2.4% at 800 nm. The photocathode’s active area for imaging is set as 25×25 mm². The spatial resolution of the detector, which is determined by the readout precision of the resistive anode incorporated into the photon counting tube, is ~200 μm. Three types of specially designed lens systems (Fuji Optical Co. Ltd, Tokyo, Japan) are used for adapting three ranges of magnification of 0.33, 1.0, and 5.0. Their respective object sizes are 75×75, 25×25, and 5×5 mm. For improvement of light collection efficiency, their numerical apertures are designed, respectively, at 0.17, 0.5, and 0.65, and the lenses are reduced to six when considering transmittance. The lens system has a built-in shutter mechanism.

Output pulses from the resistive anode in the detector are fed to a position computer (IPD controller; Photek Ltd, UK) to determine the X–Y position of each photoelectron event. The data of the time interval between two successive photoelectron pulses with two-dimensional position data of each photoelectron event are transferred consecutively to a specially designed pulse interval counter that can store 27 bits of timing data simultaneously. The time resolution of data acquisition is determined by a pulse-to-pulse resolution of 10 μs. After data acquisition, data are transferred to a personal computer to reconstruct photon counting images and to analyse spatiotemporal properties. This process produces the intensity kinetics in the regions of interest (ROIs) or the time–space correlation of photoelectrons. The advantage of this system is its capability to set the arbitrary selection of spatial and temporal dimensions of the ROI after measurement. Details of the data collection circuit and data processing for spatiotemporal characterization analyses are presented elsewhere (Kobayashi et al., 1996).

General preparation of plants and viruses, and measurement procedures

The cowpea cv. Kudore-Sanjaku was used throughout the experiments. Plants were grown and assayed for CMV infection under conditions described elsewhere (Nasu et al., 1996). The yellow strain of CMV, CMV-Y, and the legume strain of CMV, CMV-L, were maintained in tobacco plants and purified as described (Nasu et al., 1996). Extracted primary 10–14-d-old leaves were used for assay by rubbing them with CMV inoculation of 50 μg ml⁻¹ of virions in potassium phosphate buffer after dusting with carborundum (#600). Preparation after leaf excision was performed under a weak red light to minimize the effects of fluorescence from leaves by excitation with a room light. Inoculated leaves were put onto a Petri dish with a damp filter paper, and then covered. The dish was mounted on a temperature- (27 °C) controlled dish holder in a light-tight sample chamber of the imaging system. Measurement was carried out continuously for 24 h after inoculation. With respect to data analysis, the first 5 h of data were omitted to eliminate the photon emission originating from delayed fluorescence.
The cell-permeable ROS scavenger Tiron (4,5-dehydroxy-1,3-benzene-disulphonic acid, disodium salt; Sigma-Aldrich Corp.) was used to examine the effect of antioxidants. Two plants were excised at the epigeal stem 24 h prior to inoculation: one was soaked in 100 μM Tiron solution; the other was soaked in water as a control. After leaf excision, viral inoculation was performed similarly on each leaf half. Both leaves were placed onto the same Petri dish for biophoton measurement.

Spectral analyses of biophoton emissions

Spectral analyses of ultraweak photon emissions were carried out using a filter-type spectrometer: a photon counter equipped with coloured glass filters with spectral resolution of 20–50 nm. Details of the system and the process for construction of the spectrum were described in a previous report (Kobayashi et al., 2001).

Results

Biophoton emission images of leaves during HR and their spatiotemporal properties

Temporal changes of biophoton emissions obtained under various treatment conditions are shown in Fig. 1. A time
sequence of photon emission images of two leaves after inoculation observed using the ×0.33 lens system are indicated in Fig. 1A. In Fig. 1A, the right half of the left leaf and the left half of the right leaf were inoculated with CMV-Y—the CMV strain eliciting HR. The left half of the left leaf was treated with virus-lacking buffer solution as a control. The right half of the right leaf was treated with CMV-L, which is the strain which does not elicit the HR. The time range indicated below the images in Fig. 1A represents the time elapsed after inoculation. The photograph in Fig. 1B shows the sample configuration. This photograph was taken 24 h after inoculation, indicating the expression of the local lesion on the leaf halves that had been inoculated with CMV-Y. From Fig. 1A, two regions where CMV-Y was inoculated show temporal augmentation of localized ultraweak photon emission as bright spots. In contrast, the area treated with CMV-L and buffer showed no remarkable changes in intensity. Six independent experiments in the same conditions were performed: similar results were obtained in each case. In Fig. 1C, time-courses of total photon emission intensity in each leaf half are shown, indicating that the intensity of CMV-Y-infected regions was enhanced markedly in photon emission from 10 h to 16 h after infection, which preceded the appearance of symptoms. On the buffer-treated region, the intensity showed monotonously decreased photon emission, demonstrating the effect of delayed fluorescence from the leaves. A temporal increase of photon emission was not observed. The CMV-L-treated region also showed no remarkably greater amount of emissions than the area treated with CMV-Y. Biophoton emission images acquired during each 2 h after inoculation (Fig. 1A) also indicate the orchestrated increase of light emission from infected spots, which is maximal at 13.5 h after inoculation. As shown in the photograph taken after the measurement (24 h after the inoculation), localized lesions are recognized as dark spots. These visible lesions appeared only on the CMV-Y-treated regions. Biophoton generation in the early stage of the HR implies a relationship between the photon emission and an initial trigger leading to the HR.

Figure 2 depicts an image obtained using ×1.0 magnification comparable with the object area of 25×25 mm. Temporal changes of photon emission images are shown in Fig. 2A; they are clearly identifiable spots of photon emission. These spots were confirmed as consistent with sites of emerged lesions after the measurement (24 h after the inoculation). The time-courses of the intensity in the ROI, which was sectioned into 8×8 regions demarcated as the white lattice in Fig. 2B, are indicated in Fig. 2C along with 64 graphs of time-courses of intensity on areas placed at corresponding positions for spatiotemporal assay of emissions. The ROIs were chosen to comprise a single spot in each region. The timing of the increase and peak pattern in photon emissions is recognized as roughly parallel among selected spots, and it indicates that the timing of the appearance of the peaks is between 9 h and 19 h after inoculation.

Figure 3 portrays a microscopic biophoton emission image that shows enlarged spots of a single local lesion using a ×5.0 magnification lens system. Figure 3A shows that several spots are contained in the image, with diameters of ~1 mm. An extracted region (Fig. 3B), demarcated with the white box in Fig. 3A, was analysed to show the varied pattern of development of emission peaks in a single spot. The inner region demarcated with the white box in Fig. 3B was sectioned into 8×8 regions and spatiotemporal properties were analysed. Figure 3C shows the result obtained, indicating that although the intensity level is higher at the central region than in the peripheral region, the configuration of temporal changes of emission peaks is similar over the whole selected region within the indicated time resolution of 15 min.

**Biophoton emission spectrum during HR**

The biophoton emission spectrum during augmentation of photon emission was determined as shown in Fig. 4, representing a distinct peak at 720 nm. This spectrum is the average of those obtained within the time range of peak formation. Emission spectra obtained in the other time region were comparable before and after the emission peak. The emission peak corresponds to spontaneous photon emissions from dark-adapted leaves or isolated chloroplasts, as reported previously (Hideg et al., 1991), implying that the chlorophyll molecules are the source of photon emissions during HR.

**Effect of ROS scavenger on biophoton emission**

Figure 5 shows the effect of Tiron treatment. Lower induction of transient augmentation of biophoton emission was observed on the Tiron-treated leaf (Fig. 5A, left leaf) than on the control leaf (Fig. 5A, right leaf). Figure 5B shows time-courses of biophoton emission intensity obtained by the sum of counts in each region of inoculation. On the Tiron-treated leaf, transient generation of photon emission was diminished. After measurement,
necrotic lesions were not recognized. Four independent experiments under the same conditions were performed: similar results were obtained in each case. This result suggests that ROS generation involves the HR process and the biophoton generation process.

Discussion

Transient generation of biophotons (as a sort of ‘photon burst’) at sites corresponding to the local lesion was determined. Two-dimensional images of biophoton emissions and their time sequence during expression of the HR at infected sites depicted the synchronous emergence of photons. The time range of the peak configuration in intensity among individual spots dispersed within 10–16 h after inoculation, suggesting that the increased photon emission is attributed to the oxidative burst. A biphasic oxidative burst, characterized as rapid responses occurring
within the minutes–hours time range (phase I) and later production within hours–days (phase II), is known to appear due to fungal, bacterial, or viral elicitors (Grant and Loake, 2000; Allan et al., 2001). Although two phases of bursts play a pivotal role in induction of defence mechanisms, ROS production kinetics and ROS types and their function are thought to differ. In addition, phase I is suggested as not always correlating with plant disease, but is important as a priming of phase II. In contrast, phase II, which is specific to avirulent pathogens, correlates with plants’ resistance or susceptibility to the pathogen. The tobacco–tobacco mosaic virus (TMV) system is also reported to be accompanied by two phases of ROS production. It is suggested that oxidative phase II responses correlate with HR and cell death, in contrast to the phase I response. The latter is necessary for triggering the defence mechanism, but is insufficient for the final resistance response (Allan et al., 2001). The transient augmentation of biophoton emission observed in the present experiments is presumed to originate in ROS generation in phase II, in which ROS are postulated to serve as anti-pathogens and in host cell necrosis (Mehdy, 1994; Rusterucci et al., 1996; Baker et al., 1997; Riedle-Bauer, 2000) with an added role as signal mediators leading to the subsequent defence mechanism. For the cowpea–CMV system, the elicitor Avr gene product is known to be the 2a protein, which is a subunit of the viral replicase components of CMV. Consequently, early expression of HR is inferred to result from this gene-for-gene recognition mechanism. The timing of increased biophoton emission after inoculation, which appears from 10 h to 16 h, is inferred to be consistent with the time for replication after virus entry. Suppression of transient augmentation of biophoton emission and emergence of the HR with Tiron treatment (Fig. 5) suggests ROS involvement in biophoton emission and implies a role for ROS in triggering this. This result is inconsistent with the result of Bennett et al. (2005). However, it is speculated that the discrepancy arises from the different uptake of ROS scavengers.

Spectral data, shown in Fig. 4, show that the excited species that finally emit photons originate in chlorophyll. It can be assumed that the oxidative damage in chloroplasts is involved in the emission mechanisms. The photon emission mechanism is hypothesized to excite chlorophyll molecules directly by ROS-mediated radical reaction, or indirectly through energy transfer from other excited molecules, such as excited proteins, excited carboxyls, or singlet oxygen through specific amino acid oxidation or lipid peroxidation by ROS (Hideg et al., 1990; Hideg, 1993). Therefore, these excited molecules are also potentially non-chlorophyll emitters. However, chlorophyll is dominant in the case of leaves. The photon emission burst behaviour, which continues for only a few hours, represents the existence of a finely regulated mechanism to result in the HR process and to protect healthy cells from ROS damage. This regulation mechanism may involve the orchestration of cells that form a single visible lesion, as shown in the microscopic measurement in Fig. 3, depicting the parallel increase of photon emissions of both the central and peripheral regions in the spot. Recently, the signalling role of ROS has attracted attention in the context of programmed cell death (PCD) during HR (Levine et al., 1996; Jones, 2001). Cell death induced by HR is also recognized as a part of apoptotic cell death, in common with that found in mammals. Regarding HR, the direct toxic effect of ROS might dominantly lead to necrotic cell death. Alternatively, apoptotic cell death derived by PCD might be essential to the death process (Mittler et al., 1997; Pennell and Lamb, 1997; Mittler, 2002; Vranova et al., 2002). Some studies have concluded that both processes are involved (Breusegem et al., 2001; Houot et al., 2001). It remains unclear whether the observed photon bursts represent the result of a direct attack on the pathogen or the host cell for apoptosis by ROS. The present technique is useful to analyse spatiotemporal dynamics of biophoton emission with high sensitivity. It will serve to elucidate this mechanism further in the near future.

References

Allan AC, Lapidot M, Culver JN, Fluhr R. 2001. An early tobacco mosaic virus-induced oxidative burst in tobacco indicates extracellular perception of the virus coat protein. Plant Physiology 126, 97–108.

Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP. 1997. Signaling in plant–microbe interactions. Science 276, 726–733.

Bennett M, Mehta M, Grant M. 2005. Biophoton imaging: a nondestructive method for assaying R gene responses. Molecular Plant–Microbe Interactions 18, 95–102.

Breusegem FV, Vranova E, Dat JF, Inzé D. 2001. The role of active oxygen species in plant signal transduction. Plant Science 161, 405–414.

Devaraj B, Usa M, Inaba H. 1997. Biophotons: ultraweak light emission from living systems. Current Opinion in Solid State and Material Sciences 2, 188–193.

Grant JJ, Loake GJ. 2000. Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiology 124, 21–29.

Havaux M. 2003. Spontaneous and thermoinduced photon emission: new methods to detect and quantify oxidative stress in plants. Trends in Plant Science 8, 409–413.

Hideg E. 1993. On the spontaneous ultraweak light emission of plants. Journal of Photochemistry and Photobiology B: Biology 18, 239–244.

Hideg E, Kobayashi M, Inaba H. 1990. Ultraweak photoemission from dark-adapted leaves and isolated chloroplasts. FEBS Letters 275, 121–124.

Houot V, Etienne P, Petitot AS, Barbier S, Blein JP, Suty L. 2001. Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. Journal of Experimental Botany 52, 1721–1730.

Inaba H. 1988. Super-high sensitivity systems for detection and spectral analysis of ultraweak photon emission from biological cells and tissues. Experientia 44, 550–559.
Kobayashi M, Sasaki S, Suzuki S, Enomoto M, Ehara T. 2003. 

Karasawa A, Okada I, Akashi K, Chida Y, Hase S, Nakazawa-Nasu Y, Ito A, Ehara Y. 1999. One amino acid change in cucumber mosaic virus RNA polymerase determines virulent/avirulent phenotypes in cowpea. *Phytopathology* 89, 1186–1192.

Kobayashi M, Takeda M, Sato T, Yamazaki Y, Kaneko K, Ito KI, Kato H, Inaba H. 1999. Two-dimensional imaging of ultraweak photon emission from germinating soybean seedlings with a highly sensitive CCD camera. *Photochemistry and Photobiology* 65, 535–537.

Kobayashi M, Takeda M, Ito KI, Kato H, Inaba H. 1999a. Two-dimensional photon counting imaging and spatiotemporal characterization of ultraweak photon emission from a rat’s brain in vivo. *Journal of Neuroscience Methods* 93, 163–168.

Kobayashi M, Takeda M, Sato T, Yamazaki Y, Kaneko K, Ito KI, Kato H, Inaba H. 1999b. *In vivo* imaging of spontaneous ultraweak photon emission from a rat’s brain correlated with cerebrospinal energy metabolism and oxidative stress. *Neuroscience Research* 34, 103–113.

Kobayashi M, Usa M, Inaba H. 2001. Highly sensitive detection and spectral analysis of ultraweak photon emission from living samples of human origin for the determination of biomedical information. *Transactions of the Society of Instrument and Control Engineering* E-1, 214–220.

Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C. 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Current Biology* 6, 427–437.

Makino T, Kato K, Iyozumi H, Honzawa H, Tachiiyi, Hiramatsu M. 1996. Ultraweak luminescence generated by sweet potato and *Fusarium oxysporum* interaction associated with a defense response. *Photochemistry and Photobiology* 64, 953–956.

Mansfield JW. 2005. Biophoton distress flares signal the onset of the hypersensitive reaction. *Trends in Plant Science* 10, 307–309.

Mehdy MC. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiology* 105, 467–472.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7, 405–410.

Mittler R, Simon L, Lam E. 1997. Pathogen-induced programmed cell death in tobacco. *Journal of Cell Science* 110, 1333–1344.

Nasu Y, Karasawa A, Hase S, Ehara Y. 1999. *Cry*, the resistance locus of cowpea to cucumber mosaic virus strain Y. *Phytopathology* 86, 946–951.

Ohya T, Kurashige H, Okabe H, Kai S. 2000. Early detection of salt stress damage by biophotons in red bean seedling. *Japanese Journal of Applied Physics* 39, 3696–3700.

Pennell RI, Lamb C. 1997. Programmed cell death in plants. *The Plant Cell* 9, 1157–1168.

Riedle-Bauer R, 2000. Role of reactive oxygen species and antioxidant enzymes in systemic virus infections of plants. *Journal of Phytopathology* 148, 297–302.

Roschger P. 1991. Biophoton response of plants due to environmental stress. In: Inaba H, ed. *The research report on Inaba biophoton project*. Tokyo: Japan Science and Technology Agency, 207–216.

Rusterucci C, Stallaert V, Milat ML, Pugin A, Ricci P, Blein JP. 1996. Relationship between active oxygen species, lipid peroxidation, necrosis, and phytoalexin production induced by elicitors in Nicotiana. *Plant Physiology* 111, 885–897.

Vranova E, Inzé D, Van Breusegen F. 2002. Signal transduction during oxidative stress. *Journal of Experimental Botany* 53, 1227–1236.