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Sulfonylurea-resistant biotypes of *Monochoria vaginalis* generate higher ultraweak photon emissions than the susceptible ones

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Abstract

All living organisms spontaneously generate ultraweak photon emissions, which originate from biochemical reactions in cells. Current research uses the ultraweak photon emission from organisms as a novel indicator in nondestructive analyses of an organisms living state. This study indicates that ultraweak photon emissions from *Monochoria vaginalis* are different between resistant biotypes (R) to sulfonulurea (SU) and susceptible biotypes (S). In SU-R biotypes, distinct increases in photon emissions were observed, but there was little increase in SU-S biotypes. In addition, photon emissions from the resistant biotypes of *M. vaginalis* were suppressed by treatment with P450 inhibitors. This suggests that cytochrome P450 monooxygenase, which plays a crucial role in the metabolic detoxification of SUs, could be associated with the generation of ultraweak photon emissions. Ultraweak photon emissions have a potential use in a novel diagnosis system as an indicator in a nondestructive testing of weeds resistant to SUs.

Keywords: Cytochrome P450 monooxygenase; Herbicide resistance, *Monochoria vaginalis*, Photon counter, Sulfonylurea, Ultraweak photon emission

1. Introduction

Acetolactate synthase (ALS)[1] inhibitor-resistant (R) biotypes of weeds have increased worldwide [1], and to date, the biotypes of 95 weed species have been reported [2]. Sulfonylurea (SU) herbicides are one of the most potent ALS-inhibiting herbicides used worldwide. An SU-R weed biotype was first reported in 1990 [3, 4], and since then, the number of SU-R weed species has increased dramatically [2].

Visual effects of SU herbicides on weeds develop slowly because their target site is the inhibition of the enzyme that catalyzes the biosynthesis of branched chain amino acids [5]; hence, it takes a long time to judge the effect of SU herbicides visually. In order to construct a control strategy for SU-R weed biotypes, a rapid identification system is essential. Gerwick *et al*. [6] developed an in vivo assay method to identify SU-R biotypes, which depended on ALS activity. The method has been applied to several weed species [7-10]. In addition, a simpler method was proposed based on observations of growth inhibition of roots or shoots treated with SU herbicides [11-13].

Among such methods, it was reported the potential of ultraweak photon emission as a novel indicator of resistance biotypes[14]. Ultraweak photon emissions, commonly referred to as biophotons, are very weak light emissions from biological systems with intensities in the order of a few to hundreds of photons per second per square centimeter of surface area, and an almost continuous spectrum within the optical range of at least 200–800 nm [15,16]. Current research makes it clear that ultraweak photon emissions are associated with physiological conditions and can be an effective indicator in nondestructive analyses of organisms in a living state [17, 18]. In our previous work, it was demonstrated that the intensity of ultraweak photon emission from *Scripus juncoides* treated with SU herbicides was different between the SU-resistant biotype and susceptible biotypes, i.e. the biophoton emission intensity after SU treatment was higher in the resistant biotypes than in the susceptible biotypes [14]. However, it is unknown whether this photon emission from resistant biotypes was found in other weed species.

Although the precise molecular mechanism underlying ultraweak photon emission has not been fully clarified, it has been suggested that ultraweak photon emission occurs as a result of fluorescent substances, including unsaturated fatty acids, nucleic acids, amino acids, and polyphenols, being peroxidized and excited by reactive oxygen

[1] *Abbreviations:* ALS, acetolactate synthase; BSM, bensulfuron-methyl; DMF, *N*,*N*′-dimethyl formamide; P450, cytochrome P450 monooxygenase; R, resistant; S, susceptible; SU, sulfonylurea.

species; by energy transfer from excited carbonyl or other substances to luminescence substances. The detoxification metabolism includes oxidation-reduction reactions. In fact, it was reported that an extremely strong increase in biophoton intensity is observed when rice and barnyard grass, which has tolerance by detoxification metabolism, are treated with SU herbicide [19]. Furthermore, these photon emissions from rice and barnyard grass were suppressed when the leaf segments were treated with cytochrome P450 monooxygenase (P450) inhibitors, and it is suggested that this generation of emission is associated with P450, which is an enzyme involved in oxidative metabolism [20-24].

Therefore we hypothesize ultraweak photon emission from weed treated with SU might be associated with detoxification metabolism with P450. In this study, we studied the difference in ultraweak photon emissions between SU-R and -S biotypes of *Monochoria vaginalis*, and effect of P450 inhibitors on the ultraweak photon emissions from *M. vaginalis*.

2. Materials and methods

2.1. Plant samples

Four SU-R and four SU-S biotypes of *M. vaginalis* were collected from rice paddy fields in Japan and identified by a root bioassay using an 'Instant test-in-office kit' (DuPont Japan Ltd., Tokyo, Japan)[2]. A mutation site of an ALS gene was checked by restriction analysis and direct sequencing. Kamituneyoshi-R has a mutation with a Pro197 to Ser in *ALS3* and Zennoji-R has a mutation with a Pro197 to Ser in *ALS1*, respectively [25]. Wakamiya-R has a mutation with a Asp376 to Glu in *ALS1* [26], and Keisen-R has a mutation with a Ala205 to Val in *ALS1* (Imaizumi, unpublished). Their self-pollinated seeds were sown and cultivated in 1/5,000a Wagner pots at Kyoto University, Kyoto, Japan.

2.2. Chemicals

An SU herbicide, bensulfuron-methyl (BSM) [methyl-α-(4,6-dimethoxypyrimidin -2-yl-carbamoylsulfamoyl)-*o*-toluate] (DuPont Japan Ltd.), most commonly used in paddy fields in Japan, was used in this study.

[2] DuPont Japan Ltd., 2-11-1 Nagata-Cho, Chiyoda-Ku, Tokyo 100-6111, Japan.

Sulfometuro-metyl was purchased from the Sigma Aldrich Japan Co. (Tokyo, Japan) [3].The P450 inhibitors, malathion (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [4]were dissolved in *N N'*-dimethyl formamide (DMF; Wako Pure Chemical Co., Ltd., Osaka, Japan) [5] as 100-fold stock solutions. We confirmed that the BSM solution, P450 inhibitors and distilled water never generated photon emissions by themselves.

2.3. Apparatus for ultraweak photon emission measurements

Ultraweak photon emissions were detected with a photon counting method using a photon counter PCX-100 (Hamamatsu Photonics K.K., Hamamatsu, Japan)[6]. The PCX-100 is equipped with an R329 photomultiplier tube, which provides a spectral response from 240 to 630 nm. The usable area for measurement of Petri dishes is 16.7 cm^2. The photomultiplier moves onto 16 samples, and ultraweak photon emissions from samples were measured in rotation per 10 s at appropriate times.

2.4. Measurements of ultraweak photon emissions from sulfonylurea-resistant and -susceptible biotypes in M. vaginalis

A leaf of each plant was cut into a 5 mm square, and 0.3 g of these segments were set in Petri dishes (60 mm diameter), to which 3 ml of a 100 ppm BSM solution or distilled water (control) was added. Samples were set immediately in the photon counter PCX-100 after treatment, and the ultraweak photon emissions from each sample were continuously measured every 10 s for 42 h. The data from 24 to 40 h, in which ultraweak photon emissions were stabilized, were analyzed. All experiments were performed in triplicate.

2.5. Effect of P450 monooxygenase inhibitors on ultraweak photon emissions from M. vaginalis

Four biotypes, two SU-R (Keisen and Zennouji) and two SU-S (Maizuru and Wakamiya), were used. Leaf segments (0.3 g) of *M. vaginalis* were set in Petri dishes (60 mm diameter), to which 3 ml of a 100 ppm BSM solution or P450 inhibitors dissolved in distilled water was added. The concentrations of inhibitors used for analysis were and 150 nM of malathion. They were set in the photon counter PCX-100,

[3] Sigma Aldrich Japan Co., 2-2-24, Higashishinagawa, Shinagawa-Ku, Tokyo, 142-0002, Japan.

[4] Wako Pure Chemical Industries, 1-2, Doshomachi 3-Chome, Chuo-Ku, Osaka 540-8605, Japan.

[5] Wako Pure Chemical Industries, 1-2, Doshomachi 3-Chome, Chuo-Ku, Osaka 540-8605, Japan.

[6] Hamamatsu Photonics K. K., 5000 Hiraguchi, Hamamatsu City, Shizuoka 435-8558, Japan.

and the ultraweak photon emissions from each sample were continuously measured every 10 s for 48 h. The data from 24 to 40 h were analyzed. All experiments were performed in triplicate.

3. Results

3.1. Ultraweak photon emissions from sulfonylurea-resistant and -susceptible biotypes in M. vaginalis

Table 1 shows the amino acids of ALS at the Pro197, Ala205 and Asp376 sites encoded by ALS genes in the biotypes used. Four SU-R biotypes have different mutation site and amino acid substitution, respectively. Figure 1 shows the increases in ultraweak photon emissions from the various biotypes of *M. vaginalis*. In the four R biotypes, distinct increases in intensity of photon emissions were observed when BSM was applied compared with the water control. This higher increase was irrespective of differences in the mutation sites of the ALS genes. In contrast, increases in photon emissions in the four S biotypes were less than that in the four R biotypes. In particular, there was little difference in photon emissions between BSM application and the water control in Maizuru-S.

3.2. Effect of P450 monooxygenase inhibitors on ultraweak photon emissions from M. vaginalis

To identify the effect of P450 inhibitors on ultraweak photon emissions from *M. vaginalis* treated with BSM, pharmacological analyses were carried out using the P450 inhibitors malathion. Application of malathion alone had little effect on the ultraweak photon emissions of *M. vaginalis* (data not shown).

Figure 2 shows the increases in ultraweak photon emissions from *M. vaginalis* treated with BSM and the P450 inhibitors, and malathion. In two R biotypes, and malathion decreased ultraweak photon emissions from *M. vaginalis* treated with BSM. The increase of ultraweak photon emissions with BSM treatment in the Keisen-R biotype was suppressed by 56% with malathion. Those in Zennouji-R were suppressed by 84% with malathion. In contrast, in two susceptible biotypes, there was no definite inhibition of intensity of ultraweak photon emissions with BSM treatment.

4. Discussion

Ultraweak photon emissions were initially reported by Colli et al. [27]. Since then, there have been several reports regarding the use of ultraweak photon emission

intensity as a practical indicator incorporating simplicity and rapidity to investigate the physiological state of plants [28-32]. However, a precise mechanism underlying ultraweak photon emissions has not been fully revealed.

In this study, we demonstrated that BSM treatment induced leaf segments of *M. vaginalis* to generate ultraweak photon emissions, and increases of photon emissions were higher in SU-R biotypes than in SU-S biotypes. In addition, the difference of photon emission between R and S biotypes could be indicated in spite of variation of mutation site and amino acid. It is known that target site resistance to SU herbicides has caused by substitution of one of four amino acids (Pro197, Ala205, Asp376, Trp574) [33]. Our data in this study indicate increase of ultraweak photon emissions was observed in *M. vaginalis* with substitution in Pro197, Ala205, and Asp376. Furthermore, our previous work indicated increase of ultraweak photon emissions was observed in *Scirpus juncoides* with substitution in Pro197 and Trp574 [18]. Namely, increase of photon emission could be observed in all of four mutation sites. On the other hands, this suggests that increases of photon emissions in SU-R biotypes are not species specific, but rather a general phenomenon, and it further supports our hypothesis that ultraweak photon emissions might be a novel indicator for identifying SU-R biotypes.

To make decisions for the timely management of SU-R weed biotypes, identifying resistance is important. Therefore, several diagnoses of resistant biotypes of *M. vaginalis* have been developed. Yong et al. [34] examined several various techniques to detect SU-R biotypes, and proposed *in vitro* assays as a simple and quick method. Also, Hamamura et al. [35] and Ohno [36] proposed whole-plant bioassays as a simpler method based on observations of growth inhibition. Ultraweak photon emissions have gained considerable attention in several study fields as an extremely cheap, rapid, simple and reliable indicator with which to investigate the physiological states of plants. We propose ultraweak photon emissions as a possible novel diagnosis system for R weeds if the generation of ultraweak photon emissions is correctly associated with herbicidal selectivity.

At present, the mechanism of generation of photon emissions depending on BSM treatment is not fully clear. In the previous study, however, we reported that the ultraweak photon emissions from rice and barnyard grass, which are resistant to SUs, were suppressed by P450 inhibitor treatments [19]. Therefore, we hypothesize that P450 might influence ultraweak photon emission in SU-R biotypes of *M. vaginalis*.

The results presented in this study indicate that photon emissions from R biotypes of *M. vaginalis* were suppressed by P450 inhibitor treatments. Although a precise

mechanism underlying photon emissions remains largely unknown, our data showed the possibility that P450, which play a crucial role in the metabolic detoxification of SUs, may be associated with the generation of ultraweak photon emissions caused by SUs. It is well known that enzyme reactions such as oxidation by lypoxygenase and peroxidase are a source of photon emissions [37-39]. Therefore, it is possible that enzyme reactions in P450 inhibitors are directly related to photon emissions.

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Figure Legends

Fig. 1. The increases in ultraweak photon emissions of resistant and susceptible biotypes of *Monochoria vaginalis*. The increases of photon emissions were the differences in averages during 24–40 h after treatment between the SUs application and water control. Photon emissions were continuously measured with a PCX-100 multisample photon counter. Values represent the average of three replications. Bars indicate standard deviations (± SD). Different letters indicate a significant difference at the 5% level according to Tukey's Studentized Range Test.

Fig. 2. Effect of P450 inhibitors on ultraweak photon emissions from leaf segments of *M. vaginalis* treated with SU herbicides. The increases of photon emissions were the averages during 24–40 h after SU and P450 inhibitor treatment to the water control. Photon emissions were continuously measured with a PCX-100 multisample photon counter. Values represent the average of three replications. Bars indicate standard deviations (± SD). * and ** indicate the significant deiierences at $P < 0.05$ and 0.01, respectively.

Fig. 1. --- Inagaki *et al*. --- ↑

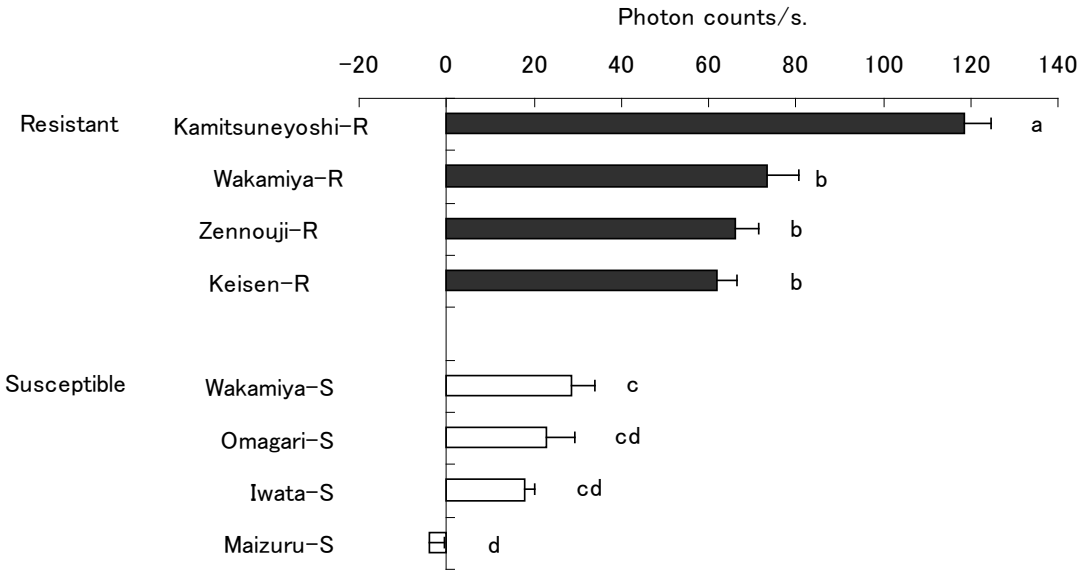

Fig. 2. --- Inagaki *et al*. --- ↑