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Note

Isolation and Identification of the Probing Stimulants in the Rice Plant for the White-back Planthopper, *Sogatella furcifera* (Homoptera: Delphacidae)

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Adult females of the white-back planthopper, *Sogatella furcifera*, showed characteristic behavior of stylet sheath deposit on a parafilm membrane when fed on a 2% aqueous crude rice leaf and stem extract containing 15% sucrose. Subsequent bioassays revealed that the butanol-soluble fraction of the extract was highly active against the insects. When the butanol fraction was chromatographed on an ODS open column and eluted in sequence with a mixture of an increasing concentration of methanol in water, the 40 % methanol fraction was separated as the most active. A further bioassay of the HPLC components in the active fraction revealed that two major components (1 and 3) stimulated the high probing activity of the white-back planthopper only when they were combined. Of the active components, one component (3) was identified to be tricin 5-*O*-glucoside by spectroscopic analyses.

Key words: *Sogatella furcifera*; probing stimulant; multi-component system; tricin 5-*O*-glucoside; rice plant

The three species of planthoppers, namely the white-back planthopper, *Sogatella furcifera*, the smaller brown planthopper, *Laodelphax striatellus*, and the brown planthopper, *Nilaparvata lugens*, are serious rice pests in the Southeast and East Asian countries. They cause huge crop yield losses by directly sucking excess plant sap from the phloem, and indirectly by transmitting rice plant diseases of economic importance. In particular, *S. furcifera* is the vector for the entomophilous fungus, *Erynia delphacis* (Entomophthorales), which has recently been isolated.[1)]

Insecticidal control of these planthoppers has been extremely difficult as a result of the emergence of several resistant biotypes. The development of alternative control techniques was therefore considered necessary.[2)]

Biological and chemical studies on the feeding of the planthopper have already identified its probing and sucking phases as being independently controlled by different sets of plant secondary chemicals.[3)] The probing stimulants in the rice plant for *N. lugens* have been isolated and identified as the combined effects of 8 flavonoid *C*-glucosides.[4,5)] The probing stimulants in the rice plant for *S. furcifera* and *L. striatellus* are, however, yet to be isolated and identified.

This paper reports the isolation of the probing stimulants in the rice plant for *S. furcifera* and their partial identification.

S. furcifera frequently showed probing behavior when given a 2% crude rice plant extract plus a 15% sucrose solution, many branched stylet sheaths (56.0 ± 0.60 points, mean ± S.E.) being observed on a parafilm membrane. On the other hand, when given only a 15% sucrose solution as a control, they did not leave any stylet sheaths (0.0 ± 0.0 point) on the membrane. This result clearly indicates that the crude rice plant extract contained the probing stimulant(s) for *S. furcifera*. The active crude rice plant extract was then partitioned into butanol and water fractions which were submitted to a bioassay. The bioassay results showed that the probing response to the butanol fraction (74.3 ± 0.65 points) was more active than that to the aqueous fraction (23.6 ± 0.43 points). When the butanol fraction was chromatographed on an ODS open column and eluted in sequence with a mixture of an increasing concentration of methanol in water, the ODS 40% methanol fraction was separated as the most active (ODS 20% methanol fr., 10.5 ± 0.45 points; ODS 40% methanol fr., 48.0 ± 0.80 points; ODS methanol fr., 19.6 ± 0.24 points). The means of the probing responses of *S. furcifera* to the crude rice plant extract, the butanol and the ODS 40% methanol fractions were not significantly different at $P = 0.01$ by a *t*-test. According to the retention times, the active

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40% ODS methanol fr. was separated into Frs. A (t_R=0–18 min) and B (t_R=18–45 min), and Fr. B was further separated into Frs. B_1 (t_R=18–28.3 min) and B_2 (t_R=28.3–45 min) by using reverse-phase HPLC (Cosmosil 5 ph column, 250 mm × 10 mm i.d.,) eluted with 20% acetonitrile in water and 1% acetic acid at a flow rate of 2 ml/min and detected at UV 254 nm (Fig. 1). Fr. B (61.1 ± 0.50 points) was more active than Fr. A (22.8 ± 0.33 points), the activity being similar to those of the ODS 40% methanol fr. and the crude rice extract. Although Fr. B_1 (35.0 ± 0.83 points) derived from Fr. B did not show the same level of activity as that of Fr. B, it was considerably more active than Fr. B_2 (9.6 ± 0.51 points) for *S. furcifera*.

From Fr. B_1, compounds **1** (t_R=18.5 min), **2** (t_R=19.0 min), and **3** (t_R=27.7 min) were isolated by preparative HPLC, and then submitted to a bioassay to give the results shown (Fig. 2). Except for compound **2**, each of the isolated compounds caused a similar level of activity as that of the parent fraction (Fr. B_1), although each of them could not recover the same level of activity as that of Fr. B. Only when all the compounds were combined, or when compounds **1** and **3** were combined, was the activity higher than that of Fr. B_1 or recovered the same level of activity as that of Fr. B. Judging from these bioassay results, it was concluded that compounds **1** and **3** were the main active compounds responsible for the probing behavior of *S. furcifera* toward the rice plant, although there were many compounds related to the probing activity, including compound **2**. The actual concentrations in the 2% crude rice extract of compounds **1** and **3** were 9.9 and 84 ppm, respectively. Their corresponding contents in 1 g of fresh leaves of the crude rice extract were 18 and 150 ppm, respectively.

Positive and negative LCMS data enabled the molecular weight of compound **3** to be found as 492. Since absorption signals were observed in the flavone and sugar regions of ^{1}H- and ^{13}C-NMR spectra of compound **3**, respectively, this compound is considered to have been a flavonoid glycoside.

Acid hydrolysis of compound **3** led to equimolar amounts of glucose and tricin, whose structures were identified by a direct comparison with their corre-

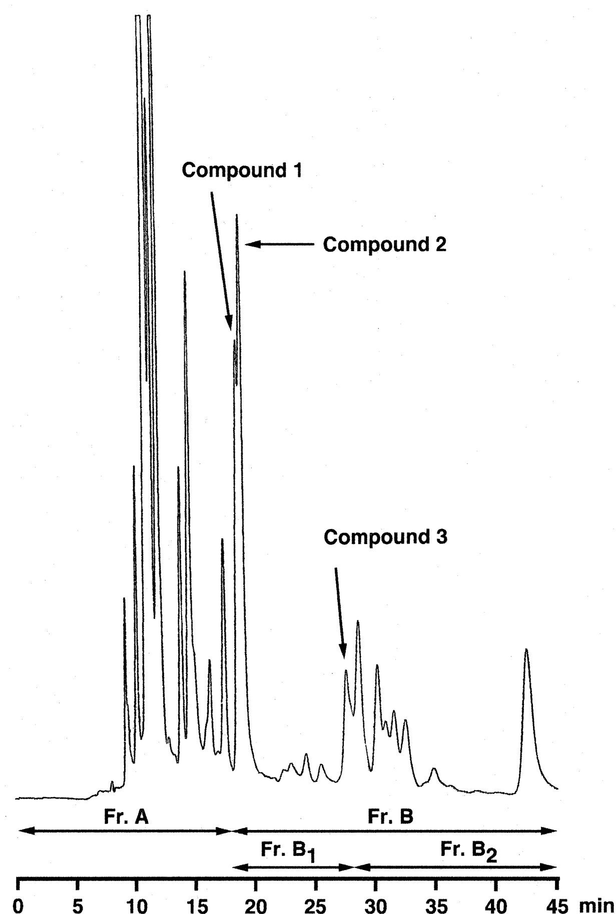

Fig. 1. HPLC Profile of the ODS 40% MeOH Fraction.

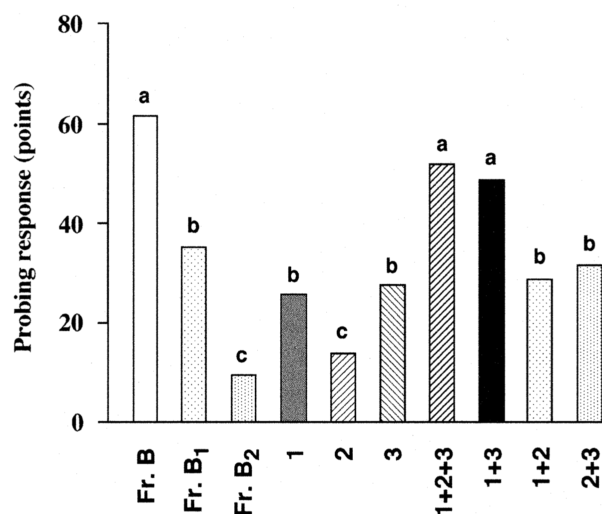

Fig. 2. Probing Response to Several Separated Fractions by HPLC. Bars with the same letters are not significantly different at P=0.01 by Anova and the t-test.

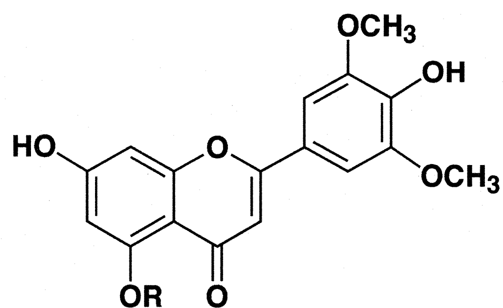

Fig. 3. Structures of Tricin and Compound **3**.

sponding authentic specimens described in the literature,[6–8] so it is reasonable that compound **3** was an *O*-flavonoid and that the glucose was attached to a certain hydroxy group within tricin.

The carbonyl carbon (177.0 ppm) shifted upfield by 4.8 ppm by comparing the ^{13}C-NMR spectra of **3** and tricin. This upfield-shift[9] is based on the disappearance of a hydrogen bond between the hydroxy group at the C-5 position, and the carbonyl carbon at the C-4 position. In other words, this shift clearly shows the absence of a hydroxy group at the C-5 position and instead it was substituted with *O*-glucoside. Therefore, it is concluded that the glucose was attached at the C-5 position. In addition to this result, since an anomeric proton of the glucose moiety had a large coupling constant ($J = 7.08$ Hz) at 4.75 ppm and specific rotation of $[\alpha]_D^{25} = +50°$, compound **3** could be identified as tricin 5-*O*-β-D-glucopyranoside, that is, tricin 5-*O*-glucoside. This compound is widely distributed in graminae plants and was initially isolated as one of several chemosystematic markers in plant taxonomy.[10,11]

This is the first report of the isolation of the probing stimulants in the rice plant for *S. furcifera*. This compound would play the role of a kairomone essential[3,4] for directing the probing sheath to the sap site in the plant. This information could be used to manipulate the feeding behavior of sucking insects and it may have the potential to be included in an integrated pest management program to effectively control sucking pests.

The structural determination of compound **1** is now in progress, and we will report its structure in the near future.

Experimental

Instruments. The LCMS data were recorded with a VG Quattro instrument (solvent system, acetonitrile:water = 50:50; flow rate, 10 μl/min with ESI-negative and ESI-positive modes). The NMR data were obtained with a JEOL JNM-LA400 spectrometer, with ^{1}H-NMR at 400 MHz and ^{13}C-NMR at100 MHz. TMS and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt were respectively used as the internal standards. The letters s, d, t and m represent singlet, doublet, triplet, and multiplet, respectively, and coupling constants (J) are given in Hz.

Insect. Stock colonies of *S. furcifera* were successively reared on rice seedlings at 25–28°C, relative humidity of 60–70% and with 16:8 (L:D) illumination.

Plant. The rice plant (cv. Toyonishiki) for extraction was cultivated without pesticidal any treatment.

Bioassay for the probing response. Five adult females of *S. furcifera* starved for 2 h were introduced into the bioassay apparatus[12] and allowed to feed on a test solution (0.45 ml) of either a rice extract plus 15% sucrose or the control (15% sucrose alone) through a parafilm membrane. The pH value of each test solution was adjusted to neutral by adding either an HCl or KOH solution.[13,14] All treatments were replicated ten times. The stylet sheaths deposited on the parafilm were observed under a microscope after being stained with a red fuchsin basic solution. After the probing sheaths had been classified according to their branching as non-branched, two-branched, three-branched, and more than four-branched, they were respectively assigned coefficients of 1, 2, 3 and 4, and the intensity of probing activity was obtained as the total number of points.[4] Frequency data were subjected to square root transformation before analysis.[15,16] All data were analysed by a one-way analysis of variance (ANOVA) and means were compared by using the t-test.[16]

Extraction procedure for the rice plant. Rice plant samples were obtained at harvest after about 14 weeks of cultivation. 2.5 kg of stems and leaves were cut into pieces (10 cm long) and completely immersed in a solution of 90% methanol in water for about 3d under darkness. This procedure was conducted three times. After filtration, the combined methanol solution was evaporated *in vacuo* to obtain a residue. After dissolving in water, the residue was defatted three times with hexane. The aqueous layer (89.8 g) was topped up to 4490 ml to obtain a 2% equivalent concentration of the "crude rice plant extract."

Isolation of the active components. This "crude rice plant extract" (89.8 g) was dissolved in distilled water (2 l) and then extracted with a water-saturated butanol (1.5 l × 3). The butanol extract (7.6 g) was chromatographed on an ODS open column (205 mm × 25 mm i.d., 50 g of Chromatorex DM1020T, 100–200 mesh; Fuji Silysia Chemical) with an increasing concentration of methanol in water to obtain the ODS 20% methanol (3.35 g), 40% methanol (2.95 g), and methanol fractions (1.31 g). The 40% methanol eluate was separated into two fractions, Fraction A ($t_R = 0$–18.0 min) and Fraction B ($t_R = 18.0$–45.0 min) by reverse-phase HPLC (Cosmosil 5 ph column, 250 mm × 10 mm i.d.), eluting with 20% acetonitrile in water and 1% acetic acid at a flow rate of 2 ml/min and detecting at UV 254 nm. Fraction B was then further separated into Fractions B_1 ($t_R = 18.0$–28.3 min) and B_2 ($t_R = 28.3$–45.0 min) by preparative HPLC. From Fr. B_1, compounds **1**, **2**, and **3** were isolated at $t_R = 18.5$ min, 19.0 min, and 27.7 min, respectively. The yield of each compound was 22.0 mg, 46.0 mg, and 73.3 mg, respectively.

Hydrolysis of compound **3**. Compound **3** (5.6 mg) was dissolved in 5 ml of $2N$ HCl and then heated at 80°C for 2.5 h. The solution was passed through a Sep-pak C_{18} ODS cartridge (Waters), eluting with 10 ml of water and then with 10 ml of methanol. D-Glucose was isolated from the aqueous eluate, and tricin was recovered from the methanol eluate.

Tricin. ^{1}H-NMR (DMSO-d_6+D_2O) δ_H: 7.35 (H-2′, 6′, 2H, s), 6.93 (H-3, 1H, s), 6.54 (H-8, 1H, s), 6.20 (H-6, 1H, s), 3.89 (H-3′, 5′-*O*-Me). ^{13}C-NMR (DMSO-d_6) δ_C: 181.7 (C-4), 164.0 (C-2), 163.6 (C-7), 161.4 (C-5), 157.3 (C-9), 148.3 (C-3′, 5′), 140.0 (C-4′), 120.5 (C-1′), 103.7 (C-10), 104.7 (C-2′, 6′), 103.6 (C-3), 98.8 (C-6), 94.1 (C-8), 56.4 (C-3′, 5′-*O*-Me).

D-Glucose. $[\alpha]_D^{25}+50°$ (c 0.1, H_2O). ^{1}H-NMR (D_2O) δ_H: 3.04–3.74 (m, β-2-6 and α-2-6), 4.48 (d, J=8.0, β-1), 5.07 (d, J=3.9, α-1).

Compound 3 (tricin 5-*O*-β-D-glucopyranoside). t_R=27.7 min (by HPLC). LCMS (ESI-negative) m/z (%): 492(M^-, 9), 491(M-H^-, 31), 285(13). LCMS (ESI-positive) m/z (%): 515 ($M+Na^+$, 17), 493($M+H^+$, 17), 331(52). ^{1}H-NMR (DMSO-d_6) δ_H: 7.30 (H-2′, 6′, 2H, s), 6.85 (H-3, 1H, s), 6.83 (H-6, 8, 2H, s), 4.75 (H-1″, 1H, d, J=7.08), 3.90 (H-3′, 5′-*O*-Me, 6H, s), 3.79 (H-6″a, 1H, d, J=11.5), 3.59 (H-6″b, 1H, dd, J=11.2, 5.1), 3.31 (H-2″, 3″, 4″, 5″, 4H, m). ^{13}C-NMR (DMSO-d_6) δ_C: 177.0 (C-4), 162.8 (C-2), 161.0 (C-7), 158.3 (C-5), 158.5 (C-9), 148.1 (C-3′, 5′), 139.4 (C-4′), 120.4 (C-1′), 108.1 (C-10), 104.4 (C-2′, 6′), 106.3 (C-3), 104.3 (C-6), 98.5 (C-8), 104.0 (C-1″), 77.5 (C-5″), 75.6 (C-3″), 73.6 (C-2″), 69.6 (C-4″), 60.8 (C-6″), 56.3 (C-3′, 5′-*O*-Me).

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