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POSSIBLE PROTECTIVE ROLE FOR 3′,4′-DIHYDROXYFLAVONES INDUCED BY ENHANCED UV-B IN A UV-TOLERANT RICE CULTIVAR

KEN R. MARKHAM, GREGORY J. TANNER,†* MERDELYN CAASI-LIT,†‡ MALCOLM I. WHITECROSS,‡ MURALI NAYUDU‡ and KEVIN A. MITCHELL

N.Z. Institute for Industrial Research and Development, P.O. Box 31310, Lower Hutt, New Zealand; †Division of Plant Industry, CSIRO, G.P.O. Box 1600, Canberra, ACT 2601, ‡Australia; Division of Botany and Zoology, The Australian National University, Canberra, ACT 0200, Australia

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Key Word Index—*Oryza sativa*; Gramineae; rice; UV-B; tolerance; 3′,4′-dihydroxyflavones; isoorientin-2″-*O*-*β*-[6-*O*-*E*-feruloylglucoside]; isoorientin-2″-*O*-*β*-[6-*O*-*E*-*p*-coumaroylglucoside].

Abstract—The investigation reported here defines the structures of the flavonoids enhanced by increased UV-B levels in a UV-tolerant rice cultivar, M202 [Caasi-Lit, M., Whitecross, M. I., Nayudu, M., Tanner, G. J., *Aust. J. Plant Physiology*, 1997, 24, 261], as *iso*orientin-2″-*O*-*β*-D-glucopyranoside, *iso*orientin-2″-*O*-*β*-[6-*O*-*E*-*p*-coumaroylglucopyranoside] and *iso*orientin-2″-*O*-*β*-[6-*O*-*E*-feruloylglucopyranoside], the *last two* being new natural products. Enhanced to a lesser extent were the *iso*vitexin analogues of the *last two*, together with *iso*scoparin-2″-*O*-*β*-[6-*O*-*E*-*p*-coumaroylglucopyranoside]. The UV-susceptible cultivar, Dular, accumulated virtually none of these compounds and no enhancement resulted from increased UV-B levels. Since the two classes of flavonoid differ very little in their ability to absorb radiation in the 290–320 nm range, these findings support recently expressed views that flavonoids may play a more subtle role in plant UV-B protection than simple UV-B screening. The presence of an *ortho*-dihydroxyl grouping in the structure is seen as of pivotal importance in that role, which may involve free radical scavenging or more efficient dissipation of UV energy. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The deleterious effect on susceptible plants of the increased UV-B radiation levels that may arise from the depletion of stratospheric ozone has attracted much attention in recent years [2, 3]. Economically important crops have been a major focus of such studies. Of the many mechanisms developed by plants to protect themselves from UV-B induced damage, perhaps the most important involves the production of UV-absorbing pigments, and in particular flavonoids [3, 4]. Flavonoids are effective absorbers of UV-B, and as they occur predominantly in the outer, epidermal layer of the leaf, are ideally sited to perform a protective, absorbing function. While flavonoids in many plants are unquestionably involved in UV-protection [4, 5] it has recently been suggested that UV-absorption may not be their sole mode of protection. In *Marchantia* [6] and *Petunia* [7] for example, significant increases in flavonoid levels were not observed under realistic UV-B enhancement regimes (compare results and light levels reported by Deckmyn *et al.* [8] with Phaseolus). Instead, a change in *the ratio* of B-ring *ortho*-dihydroxylated flavonoids to B-ring mono-hydroxylated flavonoids was observed. The relative increase in the dihydroxylated flavonoids produces no significant increase in UV-B absorptivity, and this led to the suggestion that alternative mechanisms such as radical scavenging or improved energy dissipation may account for the protective function in these examples.

Recent work by Caasi-Lit *et al.* [1] has demonstrated a wide range of UV susceptibilities in rice cultivars, from highly tolerant to highly susceptible. Not only did these cultivars differ markedly in terms of visible damage, but they also accumulated different amounts of phenolics. Thus the tolerant cultivars contained higher levels of constitutive phenolics and were capable of increasing these base

*Author to whom correspondence should be addressed.

levels by up to 250% on exposure to enhanced UV-B. There was a significant correlation between the tolerance class (based on the lack of visible symptoms of UV-B damage) and the area of the major HPLC peak in methanolic extracts of irradiated leaves. By contrast, HPLC traces of methanolic extracts from cultivars most susceptible to UV-B damage showed that these specific phenolics accumulated to a much lower extent. Other workers have shown that UV light induces a phytoalexin response in some rice cultivars, and one of these phytoalexins is the dihydroflavone, sakuranetin [9, 10], although its significance in terms of UV defense was not demonstrated. UV sensitivity in yet another cultivar, Norin 1, has been correlated with a deficiency in the cyclobutyl pyrimidine repair mechanism [11].

The results presented here follow on from the work reported by Caasi-Lit *et al.* [1] and describe the isolation and structural identification of the phenolics apparently involved in the UV-protection of tolerant rice cultivar M202. A further paper detailing the UV-B dose dependency of flavonoid accumulation in rice leaves will be published elsewhere.

RESULTS

The predominant flavonoids in *Oryza sativa* leaves appear to be flavone-*C*-glycosides. In the most comprehensive study to date, Besson *et al.* [12] defined the structures of the major plant-hopper probing stimulants in rice as the flavone-*C*-glycosides, schaftoside, neoschaftoside, carlinoside, *iso*orientin-2″-*O*-glucoside, neocarlinoside (luteolin-6-*C*-glucoside-8-*C*-arabinoside), *iso*scoparin-2″-*O*-glucoside (chrysoeriol-6-*C*-[2-*O*-glucosylglucoside]) and its 6‴-*p*-coumaroyl and -feruloyl derivatives. The tolerant rice cultivar M202 used in the present study gave HPLC profiles at 314 nm which revealed the presence of two major (flavonoid) peaks, I and II [1]. One of these, peak I, increased markedly following six days of enhanced UV-B irradiation. In contrast, the UV-susceptible cultivar, Dular, contained barely detectable levels of these compounds as constitutive components, and these levels were unaffected by enhanced UV-B irradiation. When HPLC was carried out on the M202 extract using a different chromatographic system, peak I was shown to represent two compounds, **Ia** and **Ib**, and peak II, a comparable pair of compounds, **IIa** and **IIb** (Figs 1 and 2). Subsequent work revealed that the **IIb** peak represented a mix of two components, **IIb** and **IIc**. Using this chromatographic system to assess the effect of enhanced UV-B on individual components, it became evident that the levels of compounds **Ia**, **Ib** and another higher mobility compound, **III** (Fig. 2), were most affected. After 6 days of enhanced UV-B irradiation the detected levels of compound **III** increased by varying amounts depending upon the extraction procedure. It is therefore likely to be a breakdown product. The level of compound **Ia** increased *ca.* 5× after 6 days irradiation compared to *ca.* 4× for **Ib** and only *ca.* 1.75× for **IIa** and *ca.* 1.3× for **IIb**/**IIc**. Since these increases were also UV-B dose dependent (companion paper to be published elsewhere) all five compounds were considered to be of interest with respect to the UV-resistance of the tolerant cultivar and were accordingly isolated in quantity for further study.

Irradiated rice leaves were extracted with aqueous acetone containing 0.1% ascorbate, the acetone removed *in vacuo* and the aqueous phase washed with hexane and ethyl acetate and chromatographed on Sephadex LH20. Fractions containing both peaks I and II were pooled and further chromatographed on polyamide. Fractions containing both peaks I and II were again pooled and separated by preparative reverse phase HPLC into separate fractions containing peak I or peak II. The peak I pool was further purified by chiral HPLC into two subfractions, pools Ia and Ib. The peak II pool was similarly separated into two subfractions, pools IIa and IIb. The concentration of each of the compounds in cv M202 after 8 days UV-B irradiation was estimated as approximately: **Ia** 2.0, **Ib** 0.5, **IIa** 0.19, **IIb** 0.40, **IIc** 0.007 and **IId** 0.003 mg/gfw.

Absorption spectra measured on-line during the HPLC analysis of crude extracts were all of the apigenin or luteolin flavone type, but an additional absorption band at *ca.* 320 nm was present in the spectra of all but compound **III**, suggestive of hydroxycinnamoyl acylation. This acylation was confirmed by alkaline treatment which gave products with lower retention times and on-line spectra lacking the 320 nm absorption band.

Compounds **Ia**, **Ib**, **III**

Mild alkaline treatment (see Section 4) of **Ia** and **Ib** gave the same deacylated compound. This compound was identical to **III** with respect to retention time and its absorption spectrum. Compound **III** was shown to be *iso*orientin-2″-*O*-*β*-glucoside (which has previously been identified in rice [12]) by direct comparison with an authentic sample from *Passiflora incarnata* [13]. Compounds **Ia** and **Ib** are thus identified as acylated derivatives of *iso*orientin-2″-*O*-*β*-glucoside. HPLC of the products of alkaline treatment also revealed the liberated acyl moieties, which proved identical to *p*-coumaric and ferulic acid with respect to RT (29.05 and 31.35 min respectively) and on-line absorption spectra. **Ia** and **Ib** are thereby identified as the *p*-coumaroyl and feruloyl derivatives respectively, of orientin-2″-*O*-*β*-glucoside.

Proton NMR studies on **Ia** and **Ib** at 25° in d_6-DMSO gave spectra consisting of very poorly

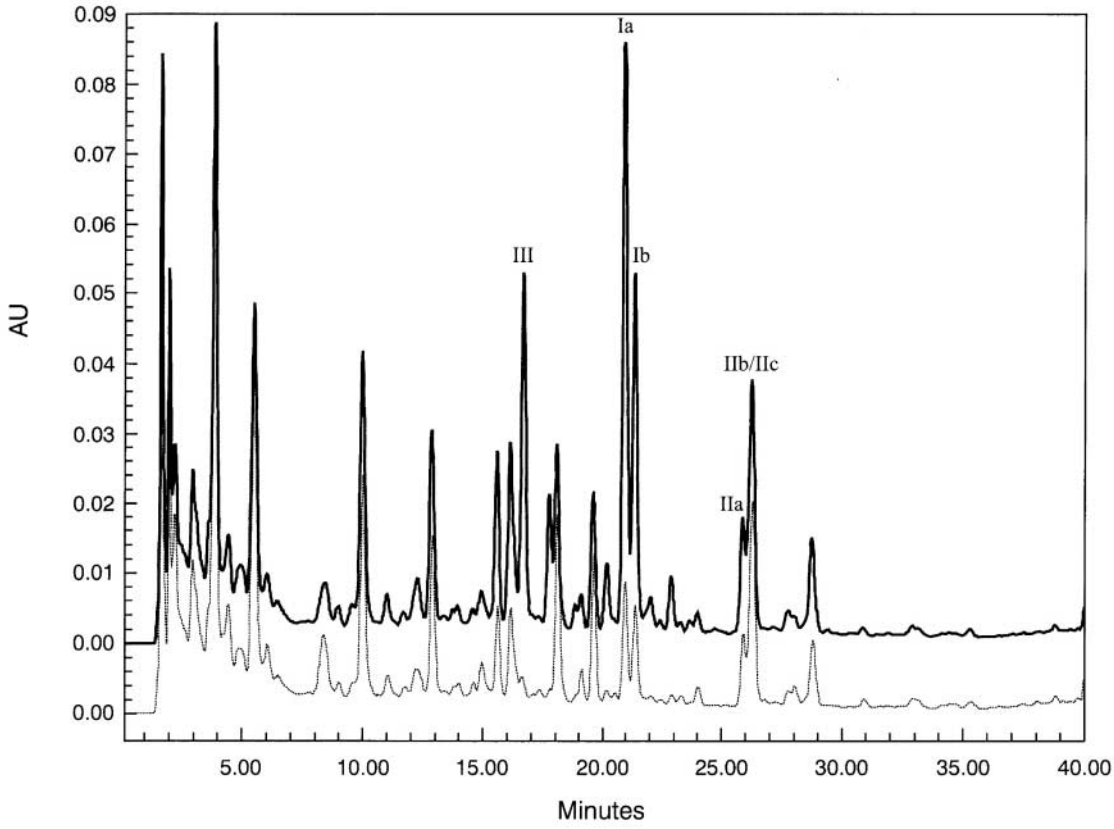
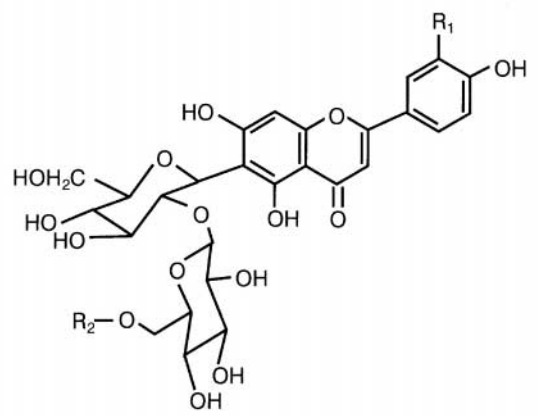


Fig. 1. HPLC comparison of the levels of constituents in tolerant rice cultivar, M202, before [dotted line] and after [solid line] UV-B enhancement. Both figures are plotted on the same scale but for clarity the traces have been offset. HPLC was carried out on a Merck LiChrospher 100RP-18 endcapped column (5 μm, 0.4 × 11.9 cm). Elution (0.8 mL/min, 30°) involved a solvent system comprising solvent A (1.5% aqueous H_3PO_4) and solvent B (HOAc:CH_3CN:H_3PO_4:H_2O, 20:24:1.5:54.5) mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% A at 39.3 min. Flavonoids were detected at 320 nm.

resolved signals. When measured at 70° however, all signals were clearly resolved; perhaps indicating the occurrence of rotational isomerism previously observed in some flavone-*C*-glycosides [14]. In the 70° spectra (Table 1) the *trans* (*E*) acyl group signals are readily identifiable and integration defines both **Ia** and **Ib** as mono-acylated compounds. The ^{13}C-NMR spectra (Table 1) confirmed the 2″ interglycosidic linkage (C-2″ at 80.2/81.5 ppm) and revealed the glucose C-6 signals at *ca.* 61.1 and 62.1 ppm. The downfield shift of the latter signal together with the upfield shift of a glucose C-5 signal to *ca.* 73.3 ppm indicates that the acyl groups are attached at one of the glucose C-6 hydroxyls in both compounds. A TOCSY experiment was used to link the H-1 of the C-glucosyl moiety to the same sugar as the nonacylated 6-hydroxyl group. Thus the anomeric H-1 signal at *ca.* δ4.75 was shown to be the H-1 of the 6-C-linked glucose through its long-range ^{1}H/^{13}C coupling to the flavonoid C-5 (160.7 ppm) and C-7 (162.9 ppm). This proton was demonstrated (TOCSY) to be part of the same sugar as produced the H-2 signal at *ca.* δ4.4 (shifted downfield by *O*-glycosylation) and the H-5 signal at *ca.* δ3.25. This H-5 signal in turn was proved by ^{1}H, ^{1}H-COSY to be coupled to the signals at *ca.* δ3.5 and δ3.76 which represent the H-6 protons on the nonacylated glucose (Table 1). Compounds **Ia** and **Ib** are accordingly assigned the structures *iso*orientin-2″-*O*-β-[6-*O*-*E*-*p*-coumaroylglucopyranoside] and *iso*orientin-2″-*O*-β-[6-*O*-*E*-feruloylglucopyranoside] respectively, both of which are new natural products.

Compounds **IIa**, **IIb** *and* **IIc**

Compounds **IIa** and **IIb** are analogues of **Ia** and **Ib** but with only 4′-*mono*-hydroxylation in the B-ring. **IIa** which was the cleaner of the two from the final HPLC clean-up, gave an ^{1}H-NMR spectrum (Table 1) identical to that of **Ia** except that the B-ring protons appeared as a pair of doublets equivalent to those of *iso*vitexin in the same solvent [15]. A single *p*-coumaroyl moiety was indicated by signals equivalent to those in the spectrum of **Ia**, and the key glucose H-1‴, H-2″, H-6″ and H-6‴ signals also matched those of **Ia**. In confirmation that the



	R_1	R_2
Ia	OH	*p*-coumaroyl
Ib	OH	feruloyl
IIa	H	*p*-coumaroyl
IIb	H	feruloyl
IIc	OMe	*p*-coumaroyl
III	OH	H

Fig. 2. Diagram showing structures of **Ia**, **Ib**, **IIa**, **IIb**, **IIc** and **III**.

sugars and sugar linkages are the same as those in **Ia**, the sugar carbon signals in the 60–83 ppm range of the ^{13}C-NMR spectrum were identical to those in the **Ia** spectrum.

The determination of the structure of **IIb** was complicated by the presence of **IIa** and **IIc** in the same fraction (determined by ^{1}H-NMR to be in the approximate ratio 1:1:1). **IIc** however was identified as *iso*scoparin-2″-*O*-*β*-[6-*O*-*E*-*p*-coumaroylglucopyranoside] from key ^{1}H-NMR signals and by cochromatography with an authentic sample isolated from rice leaves in an earlier study [12]. When the spectrum of **IIc** was removed from the composite ^{1}H-NMR spectrum it was evident that **IIa** and **IIb** have near identical spectra, apart from the signals of the acyl group. Feruloyl signals equivalent to those in **Ib** were evident at half the intensity of the *p*-coumaroyl signals (originating from **IIa** and **IIc**) in the composite spectrum. The assignments (Table 1) confirmed by ^{1}H, ^{1}H-COSY, and ^{13}C-NMR signals for all three compounds were the same as for **Ia** and **Ib** in the sugar carbon region. On the basis of the above, compound **IIb** is assigned the structure, *iso*vitexin-2″-*O*-*β*-[6-*O*-*E*-feruloylglucopyranoside].

DISCUSSION

The response of UV-tolerant rice cultivar M202 to enhanced levels of UV-B involves dramatic increases in the levels of the *iso*orientin derivatives, **Ia**, **Ib** and **III**, of between 400 and 1700%. In contrast, the *iso*vitexin equivalents, **IIa** and **IIb**, and the *iso*scoparin derivative, **IIc**, were barely affected. Since all of these compounds are virtually absent

Table 1. NMR Data for the glycosidic and acyl moieties of rice flavonoids*

Position	**Ia** (and **IIa**)		**Ib** (and **IIb**)		**IIc**
	^{13}C	^{1}H	^{13}C‡	^{1}H	^{1}H
C-glucose					
1″	71.0	4.72*d*; 10.2	71.0	4.78*d*; 9.6	4.76*d*; 10.2
2″	80.2	4.36*br*	81.5	4.4*br*	4.33*br*
3″	78.2	3.0–3.5	78.5	3.1–3.6	3.0–3.4
4″	70.1	3.0–3.5	69.9	3.1–3.6	3.0–3.4
5″	81.1	3.22*m*	81.5	3.25*m*	3.25*m*
6″	61.1	3.70*dd*; 11.8, 2, 3.48*m*	61.2	3.76*dd*; 11.4, –3.5*m*	3.71*d*; 11.7, 3.5*m*
O-glucose					
1‴	104.9	4.28*d*; 7.7	105.3	4.34*d*; 7.5	4.31d; 7.8
2‴	74.2	2.95*m*	74.2	3.0*m*	2.95*m*
3‴	76.2	3.0–3.5	76.2	3.1–3.6	3.0–3.4
4‴	69.0	3.0–3.5	68.6	3.1–3.6	3.0–3.4
5‴	73.3	3.0*m*	73.1	3.1*m*	3.0–3.4
6‴	62.1	3.88*dd*; 11.7, 3.1, 3.75*m*	62.0	3.97*dd*; 11.8, 2.7, 3.8*m*	3.85*m*
Acyl					
α	113.7	6.11*d*; 16.1	115.6†	6.22*d*; 15.8	6.09*d*; 16.1
β	143.8	7.25*d*; 15.8	145.4	7.28*d*; 16.0	7.23*d*; 15.3
1	124.9		125.3		
2	129.6	7.34*d*; 8.3	113.2	7.16*br.s*	7.32*d*; 8.5
3	115.4	6.78*d*; 8.5	149.3		6.80*d*; 8.1
4	159.3		147.6		
5	115.4	6.78*d*; 8.5	115.1†	6.83*d*; 8.3	6.80*d*; 8.1
6	129.6	7.34*d*; 8.3	122.7	6.96*d*; 7.8	7.32*d*; 8.5
CO_2R	165.7		166.0		
OMe			55.4	3.88*s*	3.85*s*

*NMR data relating to the flavonoid nucleus are presented in the Experimental section.

from the UV-susceptible cultivar studied, it is likely that this response contributes to the UV-tolerance of M202. All compounds but **III** (which is an artifact of extraction) would form ideal UV-B screens, in that they absorb strongly in the 270–360 nm region as a result of the superimposition of the hydroxycinnamic acid and flavone absorptions. Such compounds have been implicated in plant UV protection previously [16]. However, the *iso*orientin flavonoids **Ia** and **Ib** {and **III**} that are so dramatically increased on irradiation would afford the plant no better UV-B screening than do the little affected *iso*vitexin flavonoids **IIa** and **IIb** or the *iso*scoparin derivative **IIc**. The absorption spectra differ very little in the critical 290–320 nm region [17]. This casts some doubt on the proposition that the protective function of these flavonoids is solely one of UV-B screening. The structural feature distinguishing the flavonoids enhanced from those largely unaffected by UV, is the presence of a 3′,4′-*ortho*-dihydroxyl grouping in the former. Significantly, even the biosynthetically related isoscoparin derivative, **IIc**, which possesses 3′,4′-dioxygenation but lacks 3′,4′-dihydroxylation was not enhanced. Thus overall, the *ratio* of 3′,4′-dihydroxylated flavonoids to 4′-monohydroxylated flavonoids was increased following UV-B irradiation. Such a response to increased UV-B radiation has very recently been shown to be surprisingly widespread. Reuber *et al*. [5], Markham *et al*. [6] and Ryan *et al*. [7] have all reported this phenomenon in plants as diverse as a grass (rye), a liverwort (*Marchantia*) and a flowering plant (*Petunia*). It has been suggested [6, 7] that the *ortho*-dihydroxyflavonoids may be favoured as protectants against UV damage either because of their relative effectiveness as free radical scavengers of active oxygen species [18, 19], or because they are capable of more effective energy dissipation than their 4′-monohydroxy equivalents. Their superiority over 4′-mono-hydroxyflavonoids as scavengers of the tissue-damaging, oxygen-containing free radicals produced by UV-B radiation has been well documented through *in vitro* studies [20].

The investigation reported here defines the structures of the flavonoids enhanced in a UV-tolerant rice cultivar as 2″-*O*-glucosyl*iso*orientin and two new hydroxycinnamoylglucosides of isoorientin. The results suggest that the function of these compounds may not simply be one of UV-B screening. These findings reinforce recently expressed views that flavonoids may play a more subtle role in plant UV-B protection than simple UV screening, and that the presence of an *ortho*-dihydroxyl grouping may be of pivotal importance in that role.

EXPERIMENTAL

Plant materials and UV-irradiation

Seeds of two rice cultivars, M202 and Dular, were grown as described previously [1]. Sixty to 65 days after transplanting, rice plants were transferred into growth cabinets, maintained with a 12 h day and 12 h night cycle at 28/21° and 50–70% relative humidity.

Visible light was provided by 6 incandescent bulbs (100 W), and 6 cool white fluorescent tubes (400 W Philips) situated on top of a glass frame. Radiation below 290 nm was eliminated by filtering through 0.13 mm cellulose acetate which was changed every other day. UV-B was supplied by six lamps (Philips TL 20 W/12 UV). UV-B levels were measured using an IL1700 Research Radiometer with calibrated photodetector and filters (International Light, Newburyport, MA). The plant canopy was adjusted to 55–60 cm below the UV-B tubes. The UV-B irradiated plants received biologically effective fluence ($UV\text{-}B_{BE}$) at canopy height of 14–16 kJ m^{-2} d^{-1}, weighted according to the general plant action spectrum and normalised to unity at 302 nm, as previously reported [1]. PAR levels represented a daily dose of 15.1 mol m^{-2} d^{-1}, equivalent to that of a clear spring day in the Canberra region. The highest flux was in the centre of the cabinet, decreasing by 10% further away from the centre. Plants were exposed to UV-B for 8 days after being acclimated for three days.

Purification of flavonoids in HPLC peaks I and II

Preliminary attempts to purify the compounds in peak I and II were made using either 2, 5 or 30 g of UV-B irradiated rice leaves and the results from these experiments guided a larger experiment with 200 g of rice leaves. Preliminary NMR suggested that peak I and II each contained several novel acylated C-glycosylated flavonoids.

A total of 200 g f.w. of rice leaves was harvested after 6 days of UV-B exposure. The leaves were cut into small pieces and ground immediately in a total of 2 L of cold 70% MeOH, 0.1% ascorbate. The extract was filtered, concentrated under reduced pressure to 500 ml and centrifuged at 10,000*g* for 20 min. The concentrated extract was then washed with hexane and ethyl acetate, concentrated to 330 mL and stored frozen at −18°. The extract was thawed and centrifuged at 10,000*g* for 30 min and the supernatant applied to a column of Sephadex LH20 (55 × 2.5 cm) equilibrated in MilliQ water (H_2O). The column was washed with H_2O and eluted with a linear gradient from H_2O to 70% MeOH over 400 ml, followed by elution with 650 ml of 70% MeOH. Seventy fractions of 15 ml were collected at a flow rate of 1.5 mL/min. Fractions were examined by analytical HPLC for the presence of peaks I and II. Fractions 31–48

were pooled and dried under vacuum and dissolved in 5.2 mL of solvent containing *sec*-BuOH:HOAc:H_2O:$CHCl_3$ 7:1:2:1 (sBAWC) and applied to a column of polyamide (65 × 1.5 cm), eluted with s-BAWC and fractions of 15 ml collected. Fractions were examined for the presence of peak I and II using analytical HPLC and fractions 12–36 pooled.

The polyamide pool was concentrated to 3 mL under vacuum and separated by semipreparative HPLC into pools containing peak I or peak II. The purity of the pooled peak I and peak II fractions were determined using analytical HPLC as 95 and 94% respectively.

The pooled semipreparative HPLC peak I fraction was concentrated to dryness (yield 70 mg of glassy yellow compound), dissolved in 2.5 ml of ethanol and separated into pools Ia and Ib using Chiral HPLC system I. Reinjection of pool Ia showed that it contained only compound **Ia**. Reinjection of the Ib pool showed that it contained a mixture of compounds 30% **Ia** and 70% **Ib** by peak area at 314 nm. Both the Ia and Ib pools were concentrated to dryness for further NMR and HPLC studies.

The pooled semipreparative HPLC peak II fraction was concentrated to dryness (yield 20 mg of glassy yellow compound), dissolved in 1.5 mL of ethanol and separated into pools IIa and IIb using Chiral HPLC system II. Reinjection of pool IIa on Chiral HPLC system II showed that it contained a mixture of 70% compound **IIa** and 30% **IIb**. Reinjection of the IIb pool showed that it contained a mixture of 20% **IIa**, 80% **IIb**, and a trace of compound **IIc** by peak area at 314 nm. The IIa and IIb pools were concentrated to dryness for further NMR and HPLC studies.

Analytical high pressure liquid chromatography (HPLC)

Analytical HPLC for the monitoring of compound isolation was carried out using a 3 × 0.46 cm i.d. column (10 nm pore size, 3 μm packing with 12% octadecyl-coated silica) eluted at a flow rate of 2 ml/min and a temperature of 35°. The void volume of the column and system was 500 μl. The column was eluted with a linear gradient from solvent A to 70% solvent B over 5 min at 2 mL/min (solvent A = 2% (v/v) acetic acid and solvent B = methanol). The absorbance of the effluent was monitored with a single wavelength detector at 314 nm. Peaks I and II had retention times of 2.7 and 3.2 min, respectively.

Mild alkaline hydrolysis was carried out as in [20] and the products analysed on an end-capped Merck LiChrospher 100RP-18 column (5 μ; 11.9 × 0.4 cm). Injection volume was 20 μL and elution was performed using a flow rate of 0.8 mL/min at 24°. The solvents comprised water adjusted to pH 2.5 with *ortho*phosphoric acid (**A**) and acetonitrile (**B**) mixed using a linear gradient starting with 100% **A**, decreasing to 91% over the next 12 min to 87% over the next 8 min and to 67% over the next 10 min. After holding the solvent at this composition for 2 min, **A** was decreased to 57% over the next 10 min, and then held at this level until the end of the 60 min analysis. Typical retention times: *p*-coumaric acid (29.05 min), ferulic acid (31.35 min), **III** (29.98 min), **Ia** (32.74 min), **Ib** (32.90 min).

Semipreparative HPLC

80 μL aliquots were injected into a Hamilton PRP HPLC column (30 × 0.4 cm), eluted with a gradient from 40% solvent B to 80% solvent B over 15 min at a flow rate of 2 ml/min and a temperature of 35°C. Solvent A was H_2O, solvent B was MeOH. Peaks I and II eluted at 10.5 and 12.8 min, respectively.

Chiral HPLC system I

The peak I pool was injected onto a Daicel Chiral PAK-AD column (10 μm; 25 × 0.4 cm) and eluted at 1 mL/min and a temperature of 35° with 75% hexane and 25% propanol. Three compounds which absorbed at 314 nm were detected in the effluent: **Ia** (18.4 min), **Ib** (27.4 min) and **Ic** (31.8 min) in the ratio of 1:0.2:0.02 by peak area. Fractions enriched for compounds **Ia** and **Ib** respectively were obtained by repetitive injection of 40 μL aliquots of the pooled peak I fraction eluted as above at a flow rate of 1.5 mL/min. Pool Ia was collected from 8 to 14 min and pool Ib was collected from 14 to 19 min.

Chiral HPLC system II

The Peak II pool was injected onto a Daicel Chiral PAK-AD column and eluted at 1 ml/min as above except that the solvents were 70% hexane and 30% propanol. Four compounds which absorbed at 314 nm were detected in the effluent: **IIa** (5.0 min), **IIb** (6.2 min), **IIc** (7.0 min) and **IId** (9.5 min) in the ratio of 32:66:1.1:0.5 by peak area at 314 nm. Fractions enriched in compounds **IIa** and **IIb** respectively were obtained by repetitive injection of 10 μL aliquots of the pooled peak II fraction eluted as above at a flow rate of 1.5 mL/min. Pool IIa was collected from 8 to 14 min and pool IIb was collected from 14 to 19 min.

Quantification of flavonoid levels in rice leaves

A standard 1 mg/mL solution of pool Ia was used to quantify the total amount of peak I in rice leaf extracts by peak area comparison at 314 nm. The relative amounts of compounds **Ia** and **Ib** were obtained by proportioning the total amount of peak I according to the peak area ratios observed for **Ia** and **Ib** when the peak I pool was chromato-

graphed with chiral HPLC system I above. Similarly a standard solution of pool IIa was used to quantify the total amount of peak II in rice extracts. The relative amounts of compounds **IIa**, **IIb**, **IIc** and **IId** were obtained by proportioning the total amount of peak II according to the peak area ratios observed when the peak II pool was chromatographed with chiral system II above. These levels are approximations only, since compounds **Ia** and **Ib** are expected to have different extinction coefficients at 314 nm as also would compounds **IIa**, **IIb**, **IIc** and **IId**.

*NMR data for the flavone aglycone moieties of compounds **Ia**, **IIa**, **Ib**, **IIb** and **IIc***

^{1}H-NMR (δ, DMSO-d_6). **Ia**/**Ib**. 7.36*d*, 2.3 (H-2′); 7.25*dd*, 8.3, 2 (H-6′); 6.84*d*, 8.1 (H-5′); 6.41*s* (H-3); 6.38*s* (H-8). **IIa**/**IIb** 7.72*d*, 8.8 (H-2′,6′); 6.85*d*, 8.6 (H-3′5′); 6.41*s* (H-3); 6.39s (H-8). **IIc** 7.41 *br.s*. (H-2′); 7.39*dd*, 8.2, 2 (H-6′); 6.89*d*, 8.3 (H-5′); 6.57 (H-3); 6.42*s* (H-8). ^{13}C-NMR (ppm, DMSO-d_6): **Ia**/**Ib** 181.4 (C-4), 163.5 (C-2), 162.9 (C-7), 160.7 (C-5), 156.1 (C-9), 149.2 (C-3′), 145.3 (C-4′), 121.6 (C-1′), 118.5 (C-6′), 115.6 (C-5′), 113.3 (C-2′), 107.7 (C-6), 103.1 (C-10), 102.5 (C-3), 93.1 (C-8). **IIa**/**IIb** 182.0 (C-4), 163.5 br (C-2, 7,4′), 159.8 (C-5), 156.7 (C-9), 130.0 (C-2′), 128.4 (C-2′, 6′), 120.6 (C-1′), 115.9 (C-3′, 5′), 110.9 (C-6), 105.5 (C-10), 103.0 (C-3), 93.5 (C-8).

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