Induction of Hydroxycinnamic Acid Amides and Tryptophan by Jasmonic Acid, Abscisic Acid and Osmotic Stress in Barley Leaves

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The effects of jasmonic acid (JA) and abscisic aid (ABA) on secondary metabolism in barley (*Hordeum vulgare* L.) were investigated. Treatment with JA at 100 μM for 48 h induced accumulation of four compounds in barley primary leaves. The accumulation of these compounds was also observed after treatment with ABA at 100 μM. The induced compounds were identified as *p*-coumaroylputrescine, *p*-coumaroylagmatine, *p*-coumaroyl-3-hydroxyagmatine and tryptophan by spectroscopic methods. The profiles of compounds induced by application of JA and ABA were different. JA exhibited stronger inducing activity for hydroxycinnamic acid amides than ABA, while ABA was more active in tryptophan accumulation. The major hydroxycinnamic acid amides in JA- and ABA-treated leaves were *p*-coumaroylagmatine and *p*-coumaroyl-3-hydroxyagmatine, respectively. These differences suggested that JA and ABA act in distinct modes. The induction of these compounds was also observed in leaf segments treated with 1 M sorbitol and glucose. These findings suggested that JA and ABA are involved in accumulation of hydroxycinnamic acid amides and tryptophan in response to osmotic stress in barley.

Introduction

Hydroxycinnamic acid amides have been found in many plants including cereals, and their biosynthesis is considered to be regulated by both developmental and environmental factors. In barley, *p*-coumaroylagmatine occurs at a large amount in seedlings immediately after germination together with the antifungal compounds hordatine A and B (Smith and Best, 1978). Hordatine A is a dimer of *p*-coumaroylagmatine, while hordatine B is an analogous conjugate of *p*-coumaroyl and feruloylagmatines. Agmatine coumaroyl transferase (ACT, EC 2.3.1.64) activity that catalyzes the condensation between hydroxycinnamoyl-CoA and agmatine has been shown to be up-regulated in young barley seedlings (Bird and Smith, 1981; Bird and Smith, 1984). In addition, hydroxycinnamoylagmatines were reported to accumulate in seedlings infected with fungi (Peipp *et al.*, 1997; von Röpenack *et al.*, 1998), indicating the involvement of hydroxycinnamoylagmatines in active defense responses in barley. The accumulation was also induced by exogenous application of methyl jasmonate (MeJA) (Lee *et al.*, 1997).

Jasmonic acid (JA) and MeJA are widely distributed in the plant kingdom, and have been implicated in signal transduction from a variety of environmental stimuli (Creelman and Mullet, 1997; Sembder and Parthier, 1993). In barley, the action of JA has been investigated in detail in terms of induction of proteins (jasmonate-induced proteins, JIPs) (Weidhase *et al.*, 1987) and activation of genes (Lee *et al.*, 1996). Some JIPs also accumulated in leaves treated with osmoticum (Lehmann *et al.*, 1995). Application of osmoticum induced an increase in endogenous JA level (Kramell *et al.*, 2000), suggesting a role of JA as a signal mediator from osmotic stress. Abscisic acid (ABA) exhibited similar ability for inducing JIPs (Lehmann *et al.*, 1995). However, it has been suggested that ABA and JA do not act through the same signal pathway, since some jasmonate-regulated genes are not induced by application of ABA (Lee *et al.*, 1996).

Abbreviations: ABA, abscisic acid; ACT, agmatine coumaroyltransferase; JA, jasmonic acid; JIP, jasmonate-induced protein; MeJA, methyl jasmonate.

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Materials and Methods

General

NMR spectra were recorded on Bruker AC 300 and ARX 500 instruments using TMS as an internal standard. Positive ion-spray ionization mass spectra were obtained using a Perkin-Elmer-Sciex API-165 combined with an Shimadzu 10A HPLC system equipped with an ODS column (Wakosil-II 5C18 HG, 150 mm × 4.6 mm i.d.). High resolution FAB mass spectra were obtained on a JEOL JMS-600H using a glycerol matrix.

Plant material and analysis of secondary metabolites

Barley (*Hordeum vulgare* L. cv. Wasedori, Yukijirusi Shubyo, Sapporo, Japan) seeds were soaked in distilled water for 24 h to facilitate germination. The soaked seeds were then sown in wet vermiculite and maintained at 20 °C for 6 d under continuous artificial light (15 W m^{-2}) in growth chambers. The lower epidermis of 7-d-old primary leaves was peeled off, and 5 cm segments were taken 1–6 cm from the leaf tip. The segments were floated on 10 ml of solution of JA or ABA in Petri dishes with the peeled surface in contact with the solution. After incubation for an appropriate period, the leaf segments were extracted with 10 volumes of methanol for 24 h. The extracts were subjected to reversed-phase HPLC analyses [column: Wakosil II 5C18 HG, 150 mm × 4.6 mm i.d., Wako Pure Chemical, Osaka, Japan; linear gradient: 5%–10% B/A for 20 min (solvent A: H_2O containing 0.1% trifluoroacetic acid, solvent B: acetonitrile); flow rate: 0.8 ml/min; detection 280 nm].

Chemicals

Penta-*N*-acetylchitopentaose, chitopentaose and laminarihexaose were purchased from Seikagaku Kogyo, Tokyo, Japan. JA, ABA and other chemicals were obtained from Wako Pure Chemicals.

Purification of hydroxycinnamic acid amides and tryptophan from barley leaves

Barley leaf segments (140 g) treated with 100 μM JA for 72 h were extracted with 1.4 l of methanol for 24 h. After filtration, the extract was concentrated *in vacuo* to around 200 ml. The solution was washed with *n*-hexane, followed by evaporating to dryness. The residue was dissolved in water. The solution was fractionated on an ODS column (Cosmosil 75C18-OPN, Nacalai Tesque, Kyoto, Japan) equilibrated with water by stepwise elution with water, methanol-water (3:7) and methanol-water (1:1). Induced compounds were eluted in the methanol-water (3:7) fraction. This fraction was concentrated and subjected to preparative reversed-phase HPLC (column: Wakosil II 5C18 HG, 250 × 20 mm i.d.; solvent: 15% acetonitrile containing 0.5% TFA; flow rate: 6 ml/min; detection: 280 nm).

Compound **1** (10.0 mg). Ion-spray MS, *m/z* (relative intensity) 235 $[M+H]^+$ (100), 147 $[M-C_4H_{11}N_2]^+$ (5.6); ^1H NMR (300 MHz, methanol-d_4): δ 1.91 (4H, *m*, H-1, 3), 2.95 (2H, *m*, H-4), 3.34 (2H, *m*, H-1), 6.41 (1H, *d*, *J* = 15.7 Hz, H-8′), 6.80 (2H, *d*, *J* = 8.5 Hz, H-3′, 5′), 7.40 (2H, *d*, *J* = 8.5 Hz, H-1′, 6′), 7.46 (1H, *d*, *J* = 15.7 Hz, H-7′).

Compound **2** (13.0 mg). HRFAB-MS: obsd. 293.1618 (calcd for $C_{14}H_{21}N_4O_3$, $[M+H]^+$ 293.1613); ion-spray MS, *m/z* (relative intensity) 293 $[M+H]^+$ (100), 147 $[M-C_5H_{13}N_4O]^+$ (23); ^1H NMR (300 MHz, THF-d_8): δ 1.57 (1H, *m*, H-1), 1.67 (1H, *m*, H-1), 3.28 (4H, *m*, H-1, 4), 3.69 (1H, *m*, H-3), 6.37 (1H, *d*, *J* = 15.6 Hz, H-8′), 6.69 (2H, *d*, *J* = 8.2 Hz, H-3′, 5′), 7.31 (2H, *d*, *J* = 8.2 Hz, H-1′, 6′), 7.42 (1H, *d*, *J* = 15.6 Hz, H-7′), 7.44 (3H, *br s*, guanidino -NH2, =NH), 7.71 (1H, *br t*, amide NH), 8.66 (1H, *br t*, guanidino -NH), 8.74 (1H, *br s*, phenol -OH). ^{13}C NMR (75 MHz, methanol-d_4): δ 34.4 (C-1), 39.4 (C-2), 46.5 (C-4), 69.2 (C-3), 116.7 (C-3′, 5′), 118.1 (C-8′), 127.6 (C-1′), 130.6 (C-2′, 6′), 142.2 (C-7′), 158.7 (guanidino), 160.6 (C-4′), 169.8 (C-9′).

Compound **3** (33.2 mg). Ion-spray MS, *m/z* (relative intensity) 205 $[M+H]^+$ (27), 188 $[M-H_2N]^+$ (100); ^1H NMR (300 MHz, methanol-d_4): δ 3.21 (1H, *dd*, *J* = 15.3, 8.9 Hz, H-b), 3.51 (1H, *dd*, *J* = 15.3, 4.0 Hz, H-b), 3.99 (1H, *dd*, *J* = 8.9, 4.0 Hz, H-a), 7.05 (1H, *ddd*, *J* = 7.9, 6.9, 0.8 Hz, H-5), 7.13 (1H, *ddd*, *J* = 7.9, 6.9, 1.0 Hz, H-6), 7.19 (1H, s, H-1), 7.35 (1H, *dd*, *J* = 7.9, 0.8 Hz, H-7), 7.67 (1H, *dd*, *J* = 7.9, 1.0 Hz, H-4).

Compound **4** (10.8 mg). Ion-spray MS, *m/z* (relative intensity) 277 $[M+H]^+$ (100), 147 $[M-C_5H_{13}N_4]^+$ (16); ^1H NMR (300 MHz, methanol-d_4): δ 1.61–1.68 (4H, *m*, H-1, 3), 3.22 (2H, *m*, H-4), 3.31 (2H, *m*, H-1), 6.40 (1H, *d*, *J* = 15.7 Hz,

H-8′), 6.79 (2H, *d*, *J* = 8.6 Hz, H-3′, 5′), 7.40 (2H, *d*, *J* = 8.6 Hz, H-1′, 6′), 7.46 (1H, *d*, *J* = 15.7 Hz, H-7′). ^{13}C NMR (75 MHz, methanol-d_4): δ 27.2 (C-2 or 3), 27.8 (C-2 or 3), 39.7 (C-1), 42.0 (C-4), 116.8 (C-3′, 5′), 118.3 (C-8′), 127.6 (C-1′), 130.5 (C-2′, 6′), 141.9 (C-7′), 159.0 (guanidino), 160.7 (C-4′), 169.4 (C-9′).

Alkaline hydrolysis of **2**

Purified **2** (5.7 mg) was dissolved in 0.5 N NaOH (5.7 ml) and incubated for 24 h at 60 °C under nitrogen gas. The reaction mixture was neutralized with 1 N HCl, and subjected to preparative reversed-phase HPLC (column: Wakosil II 5C18 HG, 250 × 20 mm i.d.; solvent: 30% methanol containing 0.1% TFA; flow rate: 6 ml/min; detection: 280 nm) to give the hydrolysate **2a** (yield 25%).

Compound **2a**. Ion-spray MS, *m/z* (relative intensity) 251 $[M+H]^+$ (100), 147 $[M-C_4H_{11}N_2O]^+$ (71). NMR data are summarized in Table I.

Synthesis of **1** *and* **4**

Compounds **1** and **4** were synthesized from *p*-coumaroyl-*N*-hydroxysuccinimide ester and amines by the method of Negrel and Smith (1984), and the products were purified by reversed-phase HPLC. The HPLC conditions were the same as those described above.

Results

Identification of compounds induced by treatment with JA and ABA

The effects of treatment with JA and ABA on secondary metabolism in barley leaves were investigated using 6-d-old seedlings. After 48-h treatment, the leaves were extracted with methanol, and the extracts were analyzed by reversed-phase HPLC. Four peaks (**1–4**) appeared in extracts from leaves treated with JA and ABA. Compounds **1–4** were purified by two steps of reversed-phase chromatography from leaves treated with JA at 100 μM for 48 h.

Compounds **1** and **4** showed a diagnostic ion at *m/z* 147 on the ion-spray mass spectra, suggesting the presence of a *p*-coumaroyl moiety. This was also indicated by the presence of signals corresponding to a 1, 4-disubstituted benzene ring and a trans-double bond on the ^{1}H NMR spectra. The remaining signals in ^{1}H NMR spectra of **1** and **4** were assigned to putrescine and agmatine moieties, respectively, on the basis of their chemical shifts. Thus, **1** and **4** were suggested to be *p*-coumaroylputrescine and *p*-coumaroylagmatine, respectively. These compounds were chemically synthesized by transesterification of *p*-coumaric acid *N*-hydroxysuccinimide ester with putrescine and agmatine (Negrel and Smith, 1984). The spectroscopic data and retention times on reversed-phase HPLC of **1** and **4** were identical with the synthesized compounds. Compound **3** was identified as tryptophan by comparing ^{1}H NMR and ion-spray MS, and chromatographic behavior with those of the authentic compound.

The high resolution FAB-mass spectrum of **2** indicated the molecular formula of **2** to be $C_{14}H_{21}N_4O_3$, suggesting that **2** had one more hydroxyl group than *p*-coumaroylagmatine. The hydroxyl group was considered to be located on the agmatine moiety because of the presence of a *p*-coumaroyl moiety indicated by the ^{1}H NMR spectrum [a 1, 4- disubstituted benzene ring (δ_H 6.69 and 7.31) and a trans-double bond (δ_H 6.37

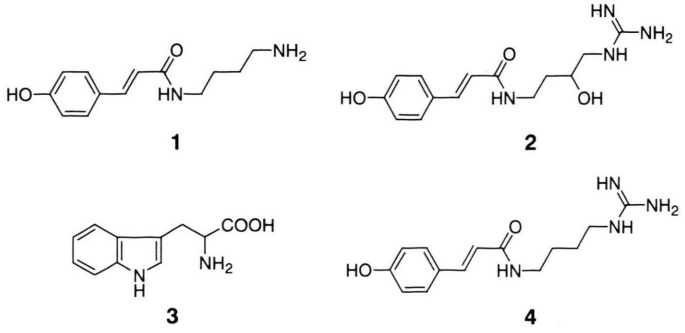

Table I. NMR data (acetonitrile-d_3) of **2a**.

Position	δH (mult., *J*, Hz)	HH COSY	NOE
H-1a	3.24 (*m*)	H-1b, 2a, 2b	
H-1b	3.54 (*m*)	H-1a, 2b	
H-2a	1.53 (*m*)	H-1a, 2b, 3	
H-2b	1.63 (*m*)	H-1a, 1b, 2a	
H-3	3.81 (*m*)	H-2a, 4a, 4b	
H-4a	2.82 (*dd*, 12.7, 9.6)	H-4b, 3	
H-4b	3.07 (*dd*, 12.7, 3.1)	H-4a, 3	
H-2′, 6′	7.43 (*d*, 8.6)	H-3′, 5′	
H-3′, 5′	6.83 (*d*, 8.6)	H-2′, 6′	
H-7′	7.46 (*d*, 15.7)	H-H-8′	
H-8′	6.39 (*d*, 15.7)	H-7′	
OH	4.47 (*s*)		
phenol-OH	7.45 (*br s*) *		
amide-OH	6.93 (*br t*)	H-1a, 1b	H-8′ (27.8%)
NH_2	7.20 (*br s*)		

* Overlapping signals of 2′, 6′ and 7′.

and 7.42)] and ion-spray mass spectrum (*m/z* 147, $[M\text{-}C_5H_{13}N_4O]^+$). This was supported by the presence of an oxygen-bearing carbon (δ_C 69.2) in the ^{13}C NMR spectrum. To identify the position of the hydroxyl group, compound **2** was subjected to alkaline hydrolysis. After 24-h reaction, the main hydrolysate **2a** was purified by preparative reversed-phase HPLC. The ion-spray mass spectrum of **2a** displayed a pseudomolecular ion at *m/z* 251, which suggested that the guanidino group was removed from 2 by hydrolysis. The ^1H NMR spectrum also showed a 1,4-disubstituted benzene ring (δ_H 6.69 and 7.31) and a trans-double bond (δ_H 6.37 and 7.42), indicating the presence of a *p*-coumaroyl moiety. The ^1H NMR spectrum exhibited four signals for active protons. Of these, a broad triplet signal (δ_H 6.93) was assigned to the amide proton on the basis of the NOE observed when the signal at δ_H 6.39 (*d*, J = 15.7 Hz, H-8′) was irradiated. A HH COSY experiment indicated the existence of the partial structure: -CO-NH-CH_2-CH_2-CH-CH_2-. From these observations **2a** was identified as *p*-coumaroyl-3-hydroxyputrescine, and thus **2** was *p*-coumaroyl-3-hydroxyagmatine.

Induction of accumulation of hydroxycinnamic acid amides and tryptophan by treatment with JA and ABA

The time course of accumulation of **1–4** was investigated after treatment with 100 μM JA and ABA (Fig. 1). The increases in amounts of **1–4** were detected 6–12 h after treatment with JA. The amounts of **1, 2** and **4** reached a maximum 72 h after treatment (285, 525 and 1580 μg/g FW, respectively). Thereafter, they decreased to 25% – 90% of the maximum by 120 h after treatment, while the amount of 3 increased throughout the

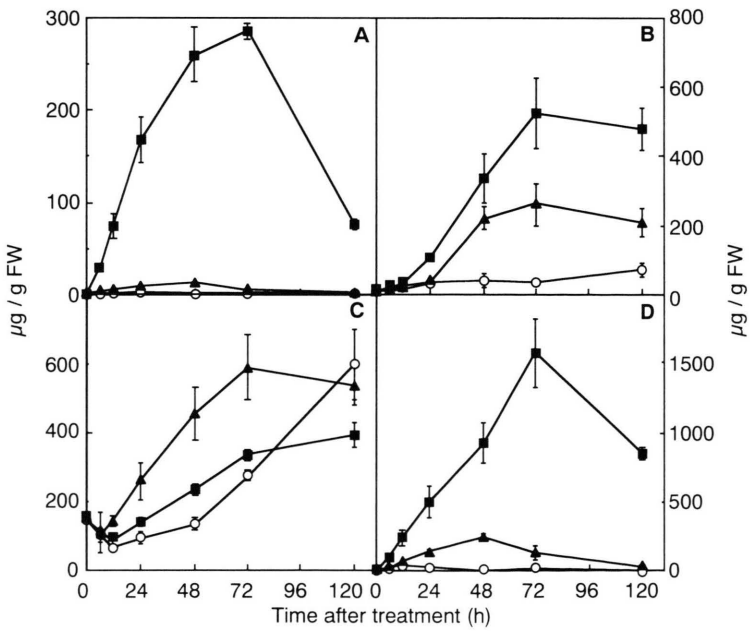

Fig. 1. Changes in amounts of *p*-coumaroylputrescine (A), *p*-coumaroyl-3-hydroxyagmatine (B), tryptophan (C) and *p*-coumaroylagmatine (D) after treatment with JA (100 μM) or ABA (100 μM). Barley leaf segments were treated with distilled H_2O (open circles), JA (solid squares) or ABA (solid triangles) for 48 h, and extracted with methanol. Each compound was quantified by reversed-phase HPLC analysis. Each data point represents the mean of three experiments with ± SD.

experimental period up to 120 h after treatment (391 μg/g FW). In ABA-treated leaves, the levels of compounds reached a maximum somewhat earlier than in leaves treated with JA; levels of compounds **1** and **4** reached the maximum 48 h after treatment (14.6 and 244 μg/g FW, respectively), while levels of **2** and **3** reached the maximum 72 h after treatment (262 and 589 μg/g FW, respectively). The composition of induced compounds in JA-treated leaves was different from that in ABA-treated leaves. Application of JA induced accumulation of larger amounts of hydroxycinnamic acid amides than ABA. Among the amides induced, the major component in JA-treated leaves was **4**, while **2** was most abundant in ABA-treated leaves. The respective maximal amounts of **1, 2** and **4** in JA treated leaves were 20-, 2.0- and 6.4-fold larger than those obtained after ABA application. On the other hand, treatment with ABA was more effective in terms of accumulation of tryptophan (**3**) than treatment with JA. The level of **3** induced by ABA was 2.1–3.4-fold larger than those in control leaves 24–72 h after treatment, while the level of **3** induced by JA was 1.2–1.8-fold higher than those in control leaves. Accumulation of hydroxycinnamic acid amides was only marginal in control leaves, while the level of **3** was increased in the late period of the experiment. This increase was probably related to the damage caused by removal of lower epidermis or detachment of leaves from seedlings, because no increase in amount of tryptophan was observed in untreated barley leaves at this stage of growth.

Figure 2 shows dependence of accumulation of **1–4** on the concentrations of JA and ABA. The amount of each compound was determined 48 h after treatment. Induction of accumulation of hydroxycinnamic acid amides was observed after treatment with JA at concentrations not less than 50 μM, and the accumulation showed saturation at around 100 μM JA. The range of concentration of ABA effective for induction of hydroxycinnamic acid amides was similar to that of JA. However, the induced amounts of hydroxycinnamic acid amides in ABA-treated leaves were smaller than those of JA-treated leaves at all concentrations tested. In contrast, accumulation of **3** was effectively induced by ABA at lower concentrations than JA. Application of ABA at 10 μM was almost sufficient for induction of the maximal amount of tryptophan. The amount of **3** increased along with the ABA concentration up to 100 μM, and decreased to 73% of the maximum at 1 mM. The accumulated amount of **3** increased along with the concentration of JA up to 1 mM.

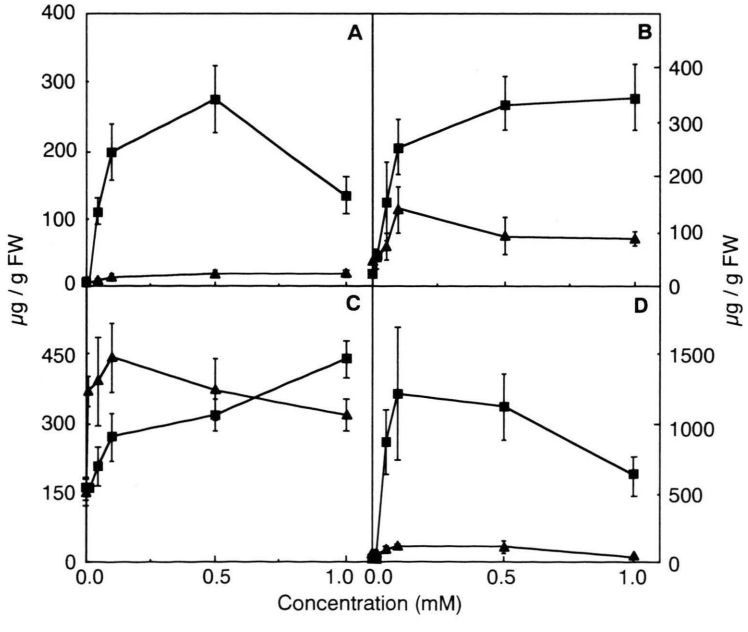

Fig. 2. Effects of concentrations of JA and ABA on the accumulation of *p*-coumaroylputrescine (A), *p*-coumaroyl-3-hydroxyagmatine (B), tryptopan (C) and *p*-coumaroylagmatine (D). Barley leaf segments were treated with JA (solid squares) and ABA (solid triangles) for 48 h. Each data point expresses the mean of three experiments with ± SD.

Effects of treatments with MeJA for 48 hr on the accumulation of **1–4** were also investigated. The induction of accumulation of **4** was observed even with 5 μM MeJA (158 μg/g FW). The amount of **4** reached a maximum at 50 μM MeJA (1689 μg/g FW), and at higher concentrations the accumulated amount somewhat decreased (1354 μg/g FW, 500 μM JA). Compounds **1** and **2** showed dose-response curves similar to **4**, the maximal amounts of **1** and **2** being 235 μg/g FW and 125 μg/g FW, respectively. The dose-response curve for the accumulation of **3** was almost identical to that obtained by treatment with JA.

Effects of osmotic stress and treatment with elicitors on accumulation of hydroxycinnamic acid amides and tryptophan

Treatment of barley leaves with sorbitol or glucose at 1 M effectively induced accumulation of **1–4** (Table II). The profiles of induced compounds were different from those observed after treatment with JA or ABA: in the osmotically stressed leaves, the respective amounts of **3** and **4** were about the same. 2-Deoxyglucose at 1 M also exhibited activity for induction of hydroxycinnamic acid amides, although the amounts were much smaller than those in leaves treated with sorbitol and glucose. However, application of NaCl at 0.5 M resulted in disappearance of **1–4**, probably because of the membrane disintegration caused by ion toxicity (Mansour *et al.*, 1993). Penta-*N*-acetylchitopentaose, chitopentaose and laminarihexaose, all of which have been shown to elicit hydroxycinnamic acid amide biosynthesis in other plants (Bordin *et al.*, 1991; Miyagawa *et al.*, 1996; Miyagawa *et al.*, 1998), were inactive with respect to induction of accumulation of **1–4**.

Distribution of hydroxycinnamic acid amides and tryptophan in young barley seedlings

It has been reported that JIPs are constitutively expressed in some tissues of young barley seedlings. In view of the similar inducibility of these compounds and JIPs, it was likely that hydroxycinnamic acid amides and tryptophan are also constitutively accumulated. Therefore, we investigated the levels of these compounds in 6-d-old seedlings. Compounds **1–4** were constitutively present at levels depending on the part of the seedling examined (Fig. 3). Compound **1** was detected only in roots. The highest levels of compounds **2, 3** and **4** were found in the scutellum, the transition region between roots and cotyledons, and coleoptile, respectively. In general, the secutellum, transition region between roots and cotyledons, coleoptile and primary leaf inside the coleoptile contained these compounds at relatively high concentrations.

Discussion

In the present study, we found that exogenous applications of ABA and JA triggered accumulation of four compounds; *p*-coumaroylputrescine

Table II. Effects of treatment with osmolytes and elicitors on the accumulation of hydroxycinnamic acid amides and tryptophan. Barley leaf segments were treated for 48 h and the amount of each compound was analyzed by HPLC.

Treatment	*p*-Coumaroyl-putrescine (**1**)	*p*-Coumaroyl-3-hydroxy-agmatine (**2**)	Tryptophan (**3**)	*p*-Coumaroyl-agmatine (**4**)
	μg/g FW	μg/g FW	μg/g FW	μg/g FW
Control	1.53	38.7	141	34.7
JA (100 μM)	261	334	212	1190
Sorbitol (1M)	56.2	119	354	457
Glucose (1M)	31.8	78.8	282	265
2-Deoxyglucose (1M)	6.17	11.0	181	97.0
NaCl (0.5 M)	n.d.	n.d.	15.6	n.d.
Penta-*N*-acetylchitopentaose (1 mM)	1.34	45.1	131	42.5
Chitopentaose (1 mM)	n.d.	24.5	76.4	14.2
Laminarihexaose (1 mM)	n.d.	19.9	107	28.3

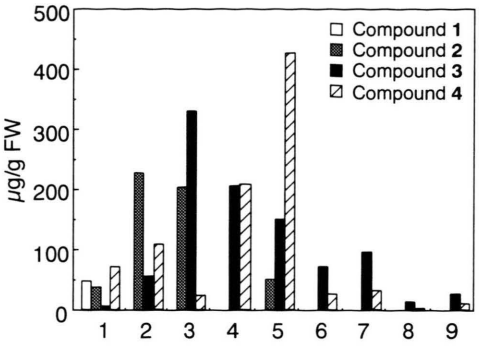

Fig. 3. Contents of hydroxycinnamic acid amides and tryptophan in different organs of 6-d-old barley seedlings. Compounds **1, 2, 3** and **4** are *p*-coumaroylputrescine, *p*-coumaroyl-3-hydroxyagmatine, tryptophan and *p*-coumaroylagmatine, respectively. Compounds were extracted from roots (1); the transition region between roots and cotyledons (2); scutellum (3); primary leaf inside the coleoptile (4); coleoptile (5); or from four 2-cm primary leaf segments starting above the coleoptile (6–9).

(**1**), *p*-coumaroyl-3-hydroxyagmatine (**2**), tryptophan (**3**) and *p*-coumaroylagmatine (**4**). Among these, it has already been reported that **1** and **4** are induced by MeJA (Lee *et al.*, 1997). However, the present study demonstrated for the first time the induced accumulation of hydroxycinnamic acid amides by application of ABA. In addition, the formation of **2** in leaves treated with ABA and JA has not been described previously. *p*-Coumaroylhydroxyagmatine was previously found to accumulate after infection with *Erysiphe graminis,* and has been suggested to play a defensive role (von Röpenack *et al.*, 1998), although its chemical structure has not been fully determined in terms of the position of the hydroxyl group on the agmatine moiety. In this study, the chemical structure was unequivocally determined as *p*-coumaroyl-3-hydroxyagmatine by hydrolysis of the compound, and NMR and ion-spray mass spectroscopy. The occurrence of γ-hydroxyagmatine derivatives has been demonstrated in other plant species (Bell, 1963a; Bell, 1963b; Hamana and Matsuzaki, 1993). Accumulation of tryptophan (**3**) was reported in barley roots infected with arbuscular mycorrhizal fungus *Glomus intraradices* (Peipp *et al.*, 1997).

The accumulation of maximal amount of main hydroxycinnamic acid amide, *p*-coumaroylagmatine, was observed following treatment with JA and MeJA at 100 μM and 50 μM, respectively. In barley, JA has been known to induce accumulation of jasmonate-induced proteins (JIPs) and expression of jasmonate-responsive genes (*jrgs*). The concentrations of JA and MeJA that are effective for the maximal induction of these reactions were reported to be 75–100 μM and 10–25 μM, respectively (Kramell *et al.*, 2000). The concentrations of JA and MeJA effective for the maximal induction of accumulation of *p*-coumaroylagmatine were close to these values. The higher activity of MeJA than JA might reflect more efficient uptake of the former. Treatment with ABA at 100 μM was demonstrated to be sufficient to induce JIPs, although the extent of induction was weak as compared to induction by treatment with 100 μM JA (Lehmann *et al.*, 1995). The concentration range of ABA effective for induction of amides also seems to be comparable for those for induction of JIPs.

In gramineous plants, accumulation of hydroxycinnamic acid amides with various amines has been increasingly documented in relation to induction by stress. For example, accumulation of feruloylagmatine is induced by low temperature in wheat (Jin and Yoshida, 2000). Hydroxycinnamoyltyramine synthesis has been shown to be activated after wounding in maize leaves (Ishihara *et al.*, 2000). Furthermore, avenanthramides, a series of hydroxycinnamic acid amides with hydroxyanthranilates, have been well characterized as oat phytoalexins (Mayama *et al.*, 1982; Mayama *et al.*, 1981). Accumulation of hydroxycinnamic acid amides is probably a general stress response in the Gramineae family, although the trigger is dependent on species. In barley, however, the production of hydroxycinnamic acid amides was not induced by wounding or elicitors that are effective for induction of amide formation in other plant systems. Since the accumulation of hydroxycinnamic acid amides has been demonstrated in pathogen-infected roots or leaves (Peipp *et al.*, 1997; von Röpenack *et al.*, 1998), barley cells probably recognize an elicitor other than those tested in this study. In this regard, JA and ABA are likely to be involved in the response to fungal infection.

Osmotic stress by 1 M sorbitol or glucose induced accumulation of hydroxycinnamic acid amides and tryptophan. The profiles of compounds induced by osmoticum displayed both characteristics of ABA and JA treatment; *p*-coumaroylag-

matine (**4**) and tryptophan (**3**) accumulated in large amounts in leaves treated with osmoticum. As the endogenous JA and ABA levels have been shown to be increased in barley leaves treated with 1 M sorbitol (Kramell *et al.*, 2000; Lehmann *et al.*, 1995), JA and ABA presumably function as signal transducers for osmotic stress.

The biological significance of induction of formation of hydroxycinnamic acid amides following treatment with osmoticum has not been elucidated. In addition to our findings, however, hydroxycinnamic acid amide seems to be accumulated in the tissues in drying processes in other plants. For instance, N-hydroxycinnamoyltyramines have been reported to accumulate in wounded tissues carrying out suberization in potato (Negrel *et al.*, 1993). In oats, avenanthramides have been found to be produced during maturation of seeds (Collins, 1989). Thus, the accumulation of hydroxycinnamic acid amides may play a role in the response to desiccation or water deficit.

Numerous toxic indole alkaloids derived from tryptophan have been identified in barley. In addition, tryptamine was identified as a stress compound in UV-irradiated barley leaves (Miyagawa *et al.*, 1994). Thus, accumulation of tryptophan in osmotically stressed leaves may be related to biosynthesis of these compounds. However, we did not find any increases in amount of other compounds after treatment with osmoticum than those described in this report. Tryptophan is biosynthesized from chorismate via anthranilate. Since two isozymes of anthranilate synthase have been suggested to have distinct metabolic roles in primary and secondary metabolism (Bohlmann *et al.*, 1996), characterization of inducible anthranilate synthase will provide insight into the biological significance of accumulation of tryptophan in barley leaves.

The manner of accumulation of hydroxycinnamic acid amides is similar to that of jasmonate-induced proteins (JIPs), particularly JIP 23 (Hause *et al.*, 1996; Lehmann *et al.*, 1995), in the following respects: 1) induction by exogenous application of JA and ABA; 2) stronger effect of JA than ABA; 3) induction by sorbitol and glucose, but little or no induction by 2-deoxyglucose or NaCl; 4) constitutive occurrence in the scutellum, transition region between roots and cotyledons, and primary leaf inside the coleoptile; and 5) high content in young barley seedlings (Bird and Smith, 1984). These similarities suggested that the enzymes responsible for the accumulation of hydroxycinnamic acid amides are under similar regulation to JIPs. On the other hand, there were several differences between accumulation of tryptophan and JIPs. ABA induced accumulation of a larger amount of tryptophan at lower concentrations than JA. In addition, accumulation of tryptophan in young seedlings has not been reported previously. Therefore, accumulation of tryptophan is considered to be controlled mainly by ABA differently from hydroxycinnamic acid amide. It has been suggested that ABA and JA do not necessarily operate through the same signaling pathway, because of the presence of jasmonate-regulated genes that are not induced by application of ABA (Lee *et al.*, 1996).

p-Coumaroylagmatine (**4**) is biosynthesized from agmatine and *p*-coumaroyl-CoA by agmatine coumaroyltransferase (ACT) in young barley seedlings (Bird and Smith, 1981). The ACT partially purified from young seedlings exhibited a strict substrate specificity to agmatine, and no conjugation reaction was detected with other structurally related amines including putrescine. *p*-Coumaroylputrescine (**1**) and *p*-couamaroyl-3-hydroxyagmatine (**2**) may thus be produced from *p*-coumaroylagmatine by hydrolysis of the guanidino moiety and hydroxylation at **3** position, respectively. Alternatively, these amides may be produced by direct condensation of *p*-coumaroyl-CoA with putrescine and hydroxyagmatine catalyzed by an isozyme of ACT that has different substrate specificity from ACT in young seedlings. The presence of several isozymes that catalyze amide formation has been found in tobacco (Fleurence and Negrel, 1989), carnation (Yang *et al.*, 1997) and oats (Matsukawa *et al.*, 2000). The differences in composition of hydroxycinnamic acid amides observed between JA- and ABA-treated leaves or among intact tissues of 6-d-old seedlings may also support the presence of isozymes of ACT. However, the possibility that the regulation of other steps in the biosynthetic pathway affects the product profile should not be excluded.

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