SUBSTRATE SPECIFICITY OF A GLUCOSYLTRANSFERASE AND AN N-HYDROXYLASE INVOLVED IN THE BIOSYNTHESIS OF CYCLIC HYDROXAMIC ACIDS IN GRAMINEAE

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Abstract—Microsomal preparations from maize seedlings exhibited N-hydroxylase activity with 2-hydroxy-1,4-benzoxazin-3-one (HBOA) as substrate, but not with its 7-methoxy analogue (HMBOA), or their corresponding 2-O-β-D-glucosides. Extracts of the hydroxamic acid (Hx)-accumulating species rye, wheat and *Hordeum lechleri*, showed UDP-glucose:Hx-glucosyltransferase activity. The hydroxamic acid, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and its 7-methoxy analogue, DIMBOA, were accepted as substrates, but not HBOA or HMBOA. The Hx-glucosyltransferase in the protein precipitate obtained between 30 and 60% ammonium sulphate saturation from either rye, wheat or *H. lechleri* had a higher V_{max} value and lower K_m value with DIMBOA as substrate. The Hx-glucosyltransferase from rye, which occurred in both roots and shoots throughout plant development, was purified 35-fold and characterized. The M_r of the enzyme was 43 000 and the isoelectric point 4.4. The K_m values for DIBOA and DIMBOA in the partly purified fraction were 73 and 82 μM, respectively, and the V_{max} for DIMBOA twice that for DIBOA. The results indicate that the glucosides of HBOA and HMBOA are not intermediates in the pathways to Hx-glucosides, and that the Hx-glucosyltransferases from species with different patterns of Hx-accumulation, are similar.

INTRODUCTION

Seedlings of several Gramineae, including rye, maize and wheat, accumulate glucosylated cyclic hydroxamic acids (Hx-Glc) derived from 2,4-dihydroxy-1,4-benzoxazin-3-one [1]. These compounds are involved in the defence of the plant against a wide variety of organisms including bacteria [2], fungi [3, 4] and a range of insects [5-7].

The presence of Hx in maize was related to resistance to the first brood of the European corn borer. This resistance was enhanced by increasing Hx concentrations in the plant through breeding [8, 9]. In the case of aphids, Hx combine within the same molecule, the properties of a behavioral pest control agent and a physiological pest control agent, thus providing the possibility of an efficient way to aphid control [10]. Hx have been proposed as targets for wheat breeding aimed at aphid-resistant cultivars [11].

The possibility of using genetic engineering techniques for manipulating the accumulation of Hx in plants could be greatly increased by knowledge about their biosynthesis and degradation. The biosynthesis of Hx has not yet been fully explored; the present information comes only from work on maize. Biogenetic studies with radiolabelled precursors indicated that the aromatic ring is derived from an intermediate in the shikimic acid pathway, and that the two heterocyclic ring carbon atoms are

derived from ribose [13]. Later work showed that radioactivity from anthranilic acid was recovered in Hx, and it was suggested, that in analogy with the biosynthesis of tryptophan, N-(5'-phosphoribosyl)anthranilate might be an intermediate in the pathway [14].

The immediate precursors of Hx are the corresponding lactams, which also accumulate in the plants, but in lower amounts than the Hx [15]. The proposed biosynthetic relationships between lactams and Hx are shown in Fig. 1. The conversion of HBOA (2-hydroxy-1,4-benzoxazin-3-one) to DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) was deduced from radiolabel incorporation experiments with [14C]HBOA [14], and was later demonstrated *in vitro* [16]. The other steps indicated in Fig. 1, i.e. the methoxylation of either HBOA to HMBOA (7-methoxy-2-hydroxy-1,4-benzoxazin-3-one) or DIBOA to DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), and the conversion of HMBOA to DIMBOA, as well as the reverse reaction, might all be deduced from radiolabel incorporation experiments [14], but none of them has as yet been confirmed *in vitro*. Figure 1 shows the compounds as aglucones, but both lactams and Hx accumulate in the intact plant as 2-O-β-D-glucosides. Hence, it is possible that the lactam glucosides rather than the aglucones are intermediates in the biosynthetic pathway.
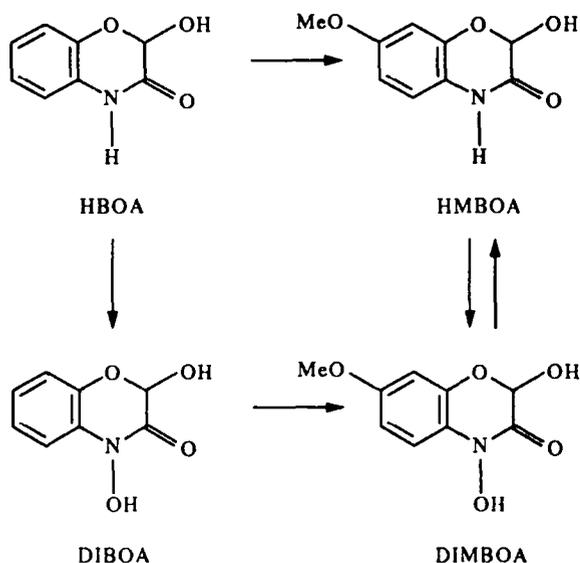
Fig. 1. Proposed biosynthetic relationships between lactams and hydroxamic acids. DIBOA is the main Hx in rye; DIMBOA the main Hx in wheat and maize. Both Hx and lactams are found in the intact plant as 2-O-β-D-glucopyranosides.

In the present work, we have focussed on the role of the lactam glucosides, and on glucosylating enzymes in the pathway to Hx glucosides. Firstly, we examined whether the lactam glucosides are substrates of an N-hydroxylation reaction. Secondly, we investigated the substrate specificity of glucosyltransferases in the pathway to Hx-glucosides. If the lactam glucosides were the substrates for the conversion of lactams into Hx, one would expect to find specific lactam glucosyltransferases. So far, only Hx-glucosylating activities have been studied. Two Hx-glucosylating enzymes were separated, one of which exhibited two times higher activity with DIMBOA than with DIBOA as substrate, and the second, 30 times higher activity with DIMBOA than with DIBOA as substrate. The apparent K_m values of the first enzyme were 174 μM for DIMBOA and 638 μM for DIBOA; and for the second enzyme, 217 μM for DIMBOA but could not be determined for DIBOA [17].

In view of the rather high apparent K_m values reported for Hx, we speculated that lactams might be better substrates for one or for both of the Hx-glucosyltransferases. Furthermore, since maize accumulates both DIBOA and DIMBOA, the above results suggested that the glucosyltransferases of Hx-accumulating plants might differ between species and be specific for the type of Hx accumulated. We have examined these possibilities by concentrating on rye, a species which accumulates DIBOA glucoside in its leaves.

RESULTS AND DISCUSSION

Substrate specificity of microsomal lactam N-hydroxylase

Microsomal preparations isolated from wheat, maize and rye, were incubated with the putative substrates,

HBOA, HMBOA, HBOA-glucoside and HMBOA-glucoside. Lactam N-hydroxylating activity could only be detected in maize microsomes, but only with HBOA as the substrate. With HBOA as substrate, an activity of 2.4 pkat mg^{-1} protein was found after 2.5 hr incubations. No product was found with any of the other substrates, even when incubations were carried out overnight, at pH 6.5, 7.5 or 8.5. Microsomal preparations from wheat (Triticum aestivum cv Kadett) or from three cultivars of rye (Secale cereale cvs Tetra Baer, Petkus II and Rogo) seedlings, did not exhibit lactam N-hydroxylating activity with any of the substrates. The possible presence of inhibitory substances in the preparation was tested by adding rye microsomes to incubations with maize microsomes. This addition, however, had no effect on the HBOA N-hydroxylating activity of maize. Therefore, at present, we cannot explain the negative results from the experiments with rye and wheat.

The results from the experiments with maize indicate that the lactam aglucones and not the lactam glucosides, are the intermediates in the pathway to Hx and, furthermore, that HBOA is a much better substrate that HMBOA. In our assays, HMBOA was not a substrate for the N-hydroxylase reaction. However, since [^{14}C]HMBOA was to some extent converted into [^{14}C]DIMBOA in incorporation experiments [14], we presume that a transformation of HMBOA to DIMBOA is possible, but that the rate of the reaction under the conditions employed in our assays, is too low to be detected.

Hx-glucosyltransferase in rye: assay conditions and expression during development

For the work on glucosyltransferases, the conditions established for enzyme assays with rye extracts insured that no β-glucosidase activity was present. Enzyme extracts from rye exhibited Hx-glucosyltransferase activity, which was completely recovered in the supernatant after centrifugation at 100 000 g, and was precipitated between 30 and 60% ammonium sulphate saturation (data not shown).

The Hx-glucosyltransferase in rye seedlings, similarly to the maize enzyme(s) showed the highest activity at pH 8.5 [17], and 45 °; half-maximal activity was obtained at ca pH 6.7. Our incubations were carried out at 37 °, which produced 92% of the activity found in incubations at 45 °.

By analogy with the maize enzymes [17], the rye Hx-glucosyltransferase activity was stimulated by addition of β-mercaptoethanol and MgCl$_2$. With 5 mM β-mercaptoethanol, activity was 400% that of controls, and with 1 mM MgCl$_2$, 145% of the controls. These compounds were, therefore, included in the assay.

Enzyme activity was detected both in roots and shoots of the seedlings (Fig. 2). Samples extracted at different times during plant development, between days 4 and 20 after sowing, gave different specific activities. In the shoots, but not in the roots, a tendency to an increase in specific activity was observed at later stages. Hx content
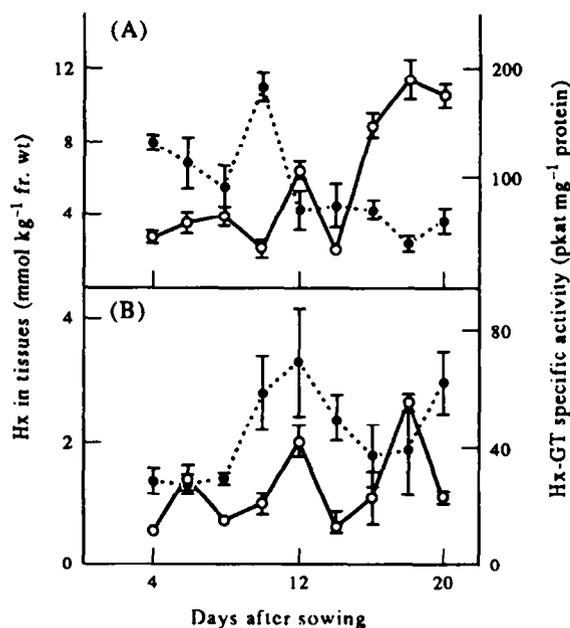
Fig. 2. Hx-glucosyltransferase activity (—O—), and Hx-content (—●—), in rye during the development of the plant. A, shoots; B, roots. Bars represent standard deviations of the mean.

Extracts from the three Hx-containing species, wheat, rye and the wild barley *Hordeum lechleri*, exhibited glucosyltransferase activity, whereas extracts from cultivated barley (*H. vulgare*) or oats (*Avena sativa*), which do not accumulate Hx, did not. This indicates that the measured activity is specific for the Hx pathway. The Hx-glucosyltransferase activity from the species which accumulate Hx, either DIMBOA (wheat) or DIBOA (rye and wild barley) as the main Hx, all showed higher maximum activity with DIMBOA than with DIBOA as substrate, and a lower apparent K_m value for DIMBOA than for DIBOA.

The results obtained, therefore, suggested that the different species contained the same type(s) of Hx-glucosyltransferase(s). Our K_m values for DIBOA of 0.23, 0.24 and 0.26 mM for rye, wheat and wild barley, respectively, were lower than the value reported for maize of 0.64 mM [17]. The difference in relative activity of our enzyme between DIBOA and DIMBOA (*ca* 150%) was however, in accordance with one of the enzymes from maize, which showed with DIMBOA as substrate *ca* 175% of the activity with DIBOA as substrate.

Crude extracts and ammonium sulphate precipitates from maize, wheat and rye were tested for lactam glucosylating activity, with both HBOA and HMBOA as the substrate. No lactam glucosylating activity was detected even after prolonged incubations (60 min for HBOA and 120 min for HMBOA) or at different pH values (6.5, 7.5 and 8.5). These results support the conclusion that the lactam aglucones are the intermediates in the pathway to Hx and, thus, the glucosylation of Hx would be the last step of the pathway. However, the question of how the lactam glucosides are formed is left open. Possibly, a reduction of Hx to lactam, as was indicated in incorporation experiments [14], is operating under certain conditions.

also showed variations during plant development, with a tendency to decrease with age in the leaves with no well defined pattern in the roots.

Substrate specificity of Hx-glucosyltransferases in different species of Gramineae

Different species belonging to the Gramineae, and with different patterns of Hx accumulation, were used as the source of glucosyltransferases. The precipitate obtained between 30 and 60% ammonium sulphate saturation was used to determine apparent K_m values and V_{max} with Hx as substrate. The results are shown in Table 1.

Partial purification of Hx-glucosyltransferase from rye and molecular properties of purified enzyme

The protein precipitate obtained between 30 and 60% ammonium sulphate saturation may contain several Hx-glucosyltransferases, and, in order to detect a possibly

Table 1. Kinetic properties of Hx-glucosyltransferase from different Gramineae species

Species	Main Hx in seedling	App. V_{max} (pkat)		App. K_m (μM)	
		DIBOA	DIMBOA	DIBOA	DIMBOA
Triticum aestivum cv Millaleu	DIMBOA	33	50	239	121
Secale cereale cv Tetra Baer	DIBOA	72	120	233	221
Hordeum lechleri PI H1310	DIBOA	22	33	264	87
Hordeum vulgare cv Libra	none	n.d.	n.d.	—	—
Avena sativa cv Nahuén	none	n.d.	n.d.	—	—

n.d.: Activity not detected (detection limit = 0.2 pkat).

Concentrations used were 0.0625, 0.125, 0.25, 0.5 and 1 mM Hx and 1 mM UDPG. K_m and V_{max} values were determined from Lineweaver–Burk plots. Correlation coefficients were 0.96 for DIMBOA and 0.98 for DIBOA in *T. aestivum*; 0.99 for DIMBOA and 0.998 for DIBOA for *S. cereale*; and 0.96 for DIMBOA and 0.97 for DIBOA for *H. lechleri*.

Table 2. Purification of Hx-glucosyltransferase from rye seedlings using DIBOA as substrate

Purification step	Total activity (nkat)	Protein (mg)	Specific activity (pkat mg^{-1} protein)	Yield (%)	Enrichment (fold)
Ammonium sulphate 60/30%	83.0	1245	67	100	1
DEAE cellulose	62.4	464	135	75	2
Hydroxyapatite	29.9	40	748	36	12
Mono Q	17.6	15	1174	21	17
Superdex 75	1.3	0.54	2375	1.6	35

Starting material: 288 g fr. wt of leaves of 14-day-old rye seedlings (*S. cereale* cv Tetra Baer).

species-specific one, the preparation from rye was subjected to several purification steps, resulting in a 35-fold purification. At the degree of purity achieved, the enzyme was stable. The purified preparation retained 70% of its initial activity for as long as three months, when kept at $-20°$ in 20 mM Tris–HCl buffer pH 7, containing 10% glycerol. The results from the partial purification are shown in Table 2. Throughout the whole procedure only one peak of Hx-glucosyltransferase was observed.

An aliquot of the Mono Q fraction was used to estimate the isoelectric point by means of chromatofocussing in a Mono P column. The analysis indicated an isoelectric point at pH 4.4. Gel filtration through Superdex 75 allowed the estimation of the M_r of the protein as 43 000.

The purified Hx-glucosyltransferase was characterized with regard to kinetic properties (Table 3). The Hx-glucosyltransferase did not glucosylate the lactams HBOA or HMBOA, nor the structurally related hydroxycoumarin, esculetin.

The apparent K_m values obtained with the partially purified enzyme were lower than those obtained using the

Table 3. Kinetic properties of partly purified Hx-glucosyltransferase from rye

Substrate	V_{max} (nkat)	K_m (μM)
DIBOA*	8.2	73
DIMBOA*	17.2	82
HMBOA†	n.d.	n.d.
HBOA†	n.d.	n.d.
Esculetin	n.d.	n.d.

n.d.: Activity not detected (detection limit $= 0.2$ pkat).

*The Superdex protein fraction (5 μg) was assayed with 0.0625, 0.125, 0.25, 0.5 and 1 mM of benzoxazinone and 1 mM UDPG.

†The Superdex protein fraction (5 μg) was assayed for 30 min at 37° as described, or 600 μg of the 60/30% ammonium sulphate fraction were assayed for 60 min at 37°, and at pH 6.5, 7.5 and 8.5.

The apparent K_m and V_{max} values were calculated from Lineweaver–Burk plots. Correlation coefficients for fits were 0.91 for DIMBOA, 0.99 for DIBOA and 0.95 for UDPG.

protein precipitate obtained between 30 and 60% ammonium sulphate saturation, indicating that the purification procedure had released interfering substances. The purified fraction showed a slightly lower apparent K_m value for DIBOA than for DIMBOA as substrate, but still had twice as high activity with DIMBOA than with DIBOA as substrate.

In conclusion, our results show that the Hx-glucosyltransferase is specific for Hx. They do not support the intermediacy of lactam glucosides in the biosynthesis of Hx glucosides. Furthermore, they do not support the concept that Hx-accumulating species contain glucosyltransferases specific for the type of Hx accumulated by the plant. The differences in apparent K_m values might be due to the interference of other compounds in the preparations, since neither previous workers [17] nor us, have purified any Hx-glucosyltransferase to homogeneity.

EXPERIMENTAL

Plant material. The following species were used in this study: *Z. mays* L. cv T 555; *S. cereale* L. cvs Tetra Baer, Rogo and Petkus II; *T. aestivum* L. cvs Millaleu and Kadett; *H. vulgare* L. cv Libra; *H. lechleri* L. PI H1310 and *A. sativa* cv. Nahuén. For the studies of N-hydroxylase activity, seedlings were grown at 21° in Petri dishes on wet paper in the dark. Otherwise, seedlings were grown in soil at 25°–30° in a growth chamber with a photoperiod of 12 hr light and 12 hr darkness, at a light intensity of 7.2 klux.

Chemicals. All chemicals, with the exception of benzoxazinones, were from Sigma. DIBOA and DIMBOA were purified from Et$_2$O extracts of 7-day-old seedlings of rye and maize, respectively, as described in ref. [18]. The corresponding glucosides were obtained from MeOH extracts of 7-day-old seedlings of rye and maize, as described in ref. [8] and purified by chromatography on DEAE Sephadex A 25 followed by chromatography on Fractogel TSK HW 40-S. Up to 10 g of the aq. extract was loaded on a 44 ml DEAE Sephadex G 25 column equilibrated and eluted with distilled H$_2$O, with a flow of 1 ml min^{-1}. The frs showing a deep purple colouration with FeCl$_3$ reagent [10% (w/v) FeCl$_3$ in 1% MeOH HCl] were pooled and lyophilized. Up to 1 g of

the lyophilized powder was then solubilized in H_2O and loaded on a 5×25 cm Fractogel TSK HW-40 S column, equilibrated and eluted with distilled H_2O with a flow of 1 ml min^{-1}. The FeCl$_3$-positive frs were pooled and lyophilized.

The lactams, HBOA and HMBOA, were synthesized as described in refs [20, 21]. The glucosylated lactams, HBOA-Glc and HMBOA-Glc, were obtained by reduction of the corresponding Hx-Glc with Zn in HOAc. The mixt. was heated at 60° with vigorous stirring and the reduction monitored with FeCl$_3$ reagent. Once the reduction was complete, the mixt. was filtered, washed with H_2O and lyophilized. The resulting powder was washed with Me_2CO, and the glucosides further purified by gel filtration on Fractogel TSK HW 40-S, as described above. The chromatographic sepn was monitored by HPLC. The identity of each compound was verified by NMR spectra taken at 300 MHz.

Extraction and analysis of Hx. Root or shoot tissue (0.2–0.4 g fr. wt) was macerated and extracted as described in ref. [22]. The content of Hx was analysed using HPLC as described below.

Enzyme extraction. All steps were carried out at 4°. In the studies of enzyme activity during development, up to 1.5 g fr. wt of shoot or root tissue were macerated with sand using a mortar and pestle, in up to 2.5 ml g^{-1} fr. tissue of homogenization buffer [0.1 M Tris–HCl pH 7.5, containing 5 mM β-mercaptoethanol 1 mM MgCl$_2$ and 30% (w/w) PVPP (buffer A)] and then filtered through cheesecloth. The homogenate was centrifuged at 19 000 g for 45 min and the supernatant filtered through Sephadex G 25 columns equilibrated with 20 mM Tris–HCl pH 7, containing 10% (v/v) glycerol (buffer B).

For studies of Hx-glucosyltransferase activities in different species, shoot tissue was used at days 6–14 after sowing, depending on plant development. Shoot tissue (15 g fr. wt) was homogenized in up to 25 ml g^{-1} fr. tissue of buffer A, as described above. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 40 000 g for 20 min. The proteins present in the supernatant were pptd with a two-step (30 and 60%) $(NH_4)_2SO_4$ procedure. The ppt. between 30 and 60% satn was resuspended in a small vol. of buffer B and filtered through Sephadex G 25 columns equilibrated with the same buffer. The prepns were used immediately for determination of enzyme activity, or after storage at $-20°$.

Preparation of microsomes. Microsomes were prepd from the radicles and epicotyls of 2.5- to 3.5-day-old seedlings as described in ref. [17]. The final (washed) pellet was resuspended in 20 mM Hepes buffer, pH 8.5.

Protein determinations. In the microsomal prepns, protein content was determined using the bicinchoninic acid protein assay according to the supplier's instruction (Pierce), and in other samples, protein was determined according to the Bradford procedure [23], using BSA as standard.

Enzyme assays. The N-hydroxylase assay contained 0.5 mM substrate, 2 mM β-mercaptoethanol, 0.5 mM NAD(P)H, 23 mM glucose-6-phosphate, 1.5 U glucose-6-

phosphate-dehydrogenase, 40 mM Hepes buffer pH 8.5 and 0.85–1.5 mg protein in a final vol. of 1 ml. The substrates were suspended in 0.04% (v/v) orthophosphoric acid and 1 mg ml^{-1} Tween 80, and diluted 10 times in the assay. Incubations were carried out at 37° with vigorous agitation, usually for 2.5 hr. In some expts (see Results), incubations were carried out overnight, and with other buffers (75 mM Bis–Tris pH 6.5; 75 mM Tris–HCl pH 7.5), and/or with the addition of NADH. Incubations containing lactam glucosides were stopped by the addition of 4 ml of CHCl$_3$–MeOH–HOAc (200:100:3); after vigorous shaking, the mixt. was centrifuged at 13 000 g for 3 min (Folch extraction). Part of the upper (MeOH–H_2O) phase was analysed using HPLC as described below. The incubations containing the lactam aglucones were stopped with HCl (60 mM final concn) and processed as described in ref. [16].

Hx-glucosyltransferase assays contained 1 mM UDPG, 0.5 mM DIBOA, or other substrates as indicated (solubilized in 0.04% orthophosphoric acid), 1 mM MgCl$_2$, 5 mM β-mercaptoethanol, 50 mM Tris–HCl pH 8.5 and 0.1 mg protein in a final vol. of 0.1 ml. For assays at pH below 8.5, castanospermine at 0.1 mM final concn was used in order to inhibit β-glucosidases. All assays were carried out at 37° in duplicate and with controls where UDPG or substrates were omitted. Incubations with extracts from *A. sativa* and *H. vulgare* contained 0.2 mg protein and were allowed to proceed for 105 min. Incubations with esculetin were carried out for 40 min with 0.6 mM substrate (solubilized in H_2O) and 0.6 mg of the ppt. obtained between 30 and 60% $(NH_4)_2SO_4$ satn or 2.25 μg of the semi-purified protein (fr. eluted from the Superdex 75 column, see Table 2) in the assay. Incubations with lactams were carried out for 60 min with 0.5 mM substrate (solubilized in 0.04% orthophosphoric acid) and with 0.1 mg protein in the assay. All other incubations were carried out for 5–20 min (reaction was at initial velocity). The incubations were stopped by a Folch extraction and the aq. phase analysed by HPLC as described below.

Purification of Hx-glucosyltransferase from rye. Up to 0.6 g of protein $(NH_4)_2SO_4$ ppt., obtained as described above, was loaded on a 60 ml DEAE-cellulose column, equilibrated in buffer B. Proteins were eluted with a 320 ml gradient of 0 to 0.3 M KCl in buffer B, with a flow rate of 0.5 ml min^{-1}. In the hydroxylapatite step, up to 0.2 g of protein was loaded on a 21 ml column equilibrated in 5 mM K-Pi pH 6.8, and the proteins eluted with a 200 ml gradient of 5 mM K-Pi, with a flow rate of 0.7 ml min^{-1}. For sepn on Mono Q HR 5/5 (Pharmacia), up to 50 mg of protein was loaded on a column equilibrated in buffer B and eluted with a 20 ml gradient of 0–0.35 M KCl, with a flow rate of 0.5 ml min^{-1}. Gel filtration was carried out using a Superdex 75 prep. grade column $(600 \times 16$ mm) (Pharmacia), equilibrated with 20 mM Tris–HCl pH 7, containing 0.25 M NaCl and 10% (v/v) glycerol. Proteins (2 mg, 1 mg ml^{-1}) were loaded and eluted at a rate of 0.4 ml min^{-1} with frs of 2 ml being collected. During all purification steps, protein elution was monitored by measuring A at 280 nm.

Chromatofocussing was carried out using a Mono P HR 5/5 column (Pharmacia), equilibrated with 25 mM Bis–Tris–iminodiacetic acid pH 7, containing 10% (v/v) glycerol. The proteins (up to 50 mg) were eluted with 20 ml of polybuffer 74-iminodiacetic acid pH 4, containing 10% glycerol (v/v) at a flow rate of 0.5 ml min $^{-1}$. Each fr. (0.5 ml) was collected over 50 μl of 1 M Tris–HCl, pH 8.

HPLC analysis. Compounds were sepd on a C18 Nucleosil column (particle size 5 μM, dimensions 150 × 4.6 mm). Detection was carried out at 263 nm. After Hx-glucosyltransferase assays, peaks were resolved with linear gradients between MeOH and 0.04% (v/v) aq. orthophosphoric acid: 0–2 min: 25% MeOH; 2–2.5 min: 25–50% MeOH; 2.5–3 min: 50–25% MeOH; 3–7 min: 25% MeOH, with a flow of 1 ml min^{-1}. The amounts of products were determined from calibration curves with standards. Detection of esculin and esculetin was at 340 nm, and elution under isocratic conditions with 1 ml min^{-1} of H$_2$O–MeOH–HOAc (69:30:1). After hydroxylation assays, peaks were resolved by eluting with H$_2$O–HOAc (9:1) at 1 ml min^{-1}. Detection of Hx in roots and shoots was at 263 nm and linear gradients between MeOH and 0.04% aq. orthophosphoric acid: 0–3 min: 35–50% MeOH; 3–4 min: 50% MeOH; 4–6 min: 50–35% MeOH; 6–9 min: 35% MeOH.

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