Levels of Maysin and Maysin Analogues in Silks of Maize Germplasm

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Maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin], the resistance factor of Zapalote Chico corn (*Zea mays* L.) silks to the corn earworm (*Helicoverpa zea* Boddie), was determined by HPLC in the silks of over 600 inbreds, populations, plant introductions (PI), and various unassigned collections. Maysin levels ranged from 0% to 0.9% fresh weight with approximately 19% of both the inbreds and populations containing maysin levels above 0.2%, a level considered to be necessary for resistance, as based on the laboratory bioassay determined level (for larval) of maysin toxicity. Over 25 lines yielded higher silk maysin than found in Zapalote Chico (0.347%). High levels of 2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)apigenin (apimaysin) were found in inbred NC7, while Tx501 contained appreciable amounts of 2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)-3′-methoxyluteolin (3′-methoxymaysin). Spectral data (^{13}C NMR and FAB-MS) are given for the characterization of maysin, apimaysin, and 3′-methoxymaysin.

INTRODUCTION

Interest in developing crops with natural resistance to insects is increasing, in response to environmental concerns and increased resistance of insects to traditional insecticides. The natural resistance of Zapalote Chico (ZC) corn silks to the corn earworm has been attributed to the presence in the silks of one major flavone called maysin [2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)-luteolin] (Waiss et al., 1979; Elliger et al., 1980a,b) (Figure 1). The identification of maysin and its methoxy analogue as potent factors in corn earworm antibiosis (Elliger et al., 1980a,b; Waiss et al., 1981) led to further studies to determine the genetic variability of available corn germplasm and the influence of environment on maysin production in corn silks (Widstrom et al., 1982, 1983). We recently developed a high-performance liquid chromatographic (HPLC) method for the determination of maysin in corn silks (Snook et al., 1989) and showed that HPLC provided a more accurate quantitation of maysin levels (Widstrom et al., 1991) than the previous spectrophotometric method (Waiss et al., 1979). Further, HPLC allowed a more complete profile of the flavone contents of the silks than was previously possible. Using HPLC, we recently identified several germplasm sources, other than ZC, that had maysin levels in their silks approaching that of ZC (Snook et al., 1989; Widstrom et al., 1991). To date, we have surveyed the maysin content of the silks from over 600 corn inbreds, populations, plant introductions (PI), and various unassigned collections. In addition to reporting many new sources of corn with high silk maysin, this paper describes the identification of several lines that contained high levels of the related flavones apimaysin and 3′-methoxymaysin.

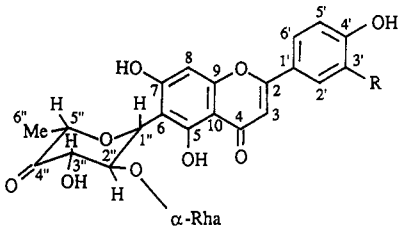

Figure 1. Structure of maysin and maysin analogues.

Table I. Numbers of Germplasm Sources vs Maysin Levels

maysin levels, % fresh wt	no. of sources		
	inbreds	populations	plant introductions[a]
>0.5	9	2	6
0.5–0.2	62	30	8
0.2–0.1	61	66	8
0.1–0.01	115	57	67
<0.01	120	9	28
total no. of lines	367	164	117

[a] Plant introductions plus unassigned sources.

MATERIALS AND METHODS

Plant Material. The majority of the plants were grown in 1989 and 1991 at the Coastal Plain Experiment Station, Tifton, GA, under standard cultural practices of fertilizer and weed control. Selected lines (Table II), for multiple-year averages, were grown from 1988 to 1992. Supplemental water was added as needed. Silks were covered to prevent pollination and were sampled when 3–5 days old.

HPLC Analysis. Sufficient numbers of plants were sampled to give approximately 30 g of silk/sample. The silks were weighed and immediately placed in 8-oz jars (Teflon-lined cap) and the jars filled with 100% MeOH (approximately 180 mL). Samples

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Table II. High Silk Maysin Corn Inbreds and Populations

Inbreds,[a] Percent Fresh Weight							
GE37	0.90	CI64	0.36[b]	L90	0.29	H49	0.25
GE80	0.85	CoeG21	0.36	9-502B	0.29	GE295	0.24
L329	0.65[b]	0-835	0.33	2-043	0.28[b]	GEC100	0.24
9-96A	0.53	0-1836	0.32[b]	49-1201B	0.28	Akd52	0.24
GE58	0.52	E2629P	0.32[b]	0-1480A	0.28	SC90	0.23
91201Y	0.52	B14 (T)	0.32[b]	GE70	0.28	Akd26	0.23
WF-038B	0.52	CI317B	0.31[b]	NC24	0.28	Tzi3D	0.23
T226	0.52	SC245A	0.31[b]	NC264	0.27	Ab604B	0.23
GT169a	0.51	A102	0.31[b]	C1-5	0.27	SC249A	0.22
Ab616	0.49	Akd24	0.31[b]	F45	0.27	0-1566	0.21
CoeG12	0.47[b]	NC64	0.31[b]	79:301-2	0.27	Akd34	0.21
Ab602	0.46[b]	CI83A	0.31[b]	9-928A	0.27	2-07A	0.21
Ab16	0.44[b]	SC249	0.30[b]	F54	0.26[b]	Ab618	0.20[b]
8940C	0.42	T315	0.30[b]	L601	0.26[b]	SC413	0.20[b]
0-909	0.39	SC102	0.30[b]	GT114	0.26[c]	NC254	0.20
SC114	0.38	GE74	0.30	W23	0.26	E2395	0.20
1-1566	0.38	GE84	0.30	Mp311	0.25	Mp305	0.20
NC45	0.36[b]	F98	0.30	GT154	0.25[b]		

Populations,[a] Percent Fresh Weight					
Oax Comp Grp.	0.56	Tbly Syn	0.32	Azteca X's	0.26
RFC-RI(C9)	0.56	Kyle Late Syn	0.30	Dial-5 P43	0.26
998x 1T#	0.45[b]	AERC(C1)	0.30[b]	Salvadoreno	0.25
2280x 1T#	0.41[b]	Gourdseed Dent	0.30	Ducle Jal	0.25
Catito Limon	0.39	Col. Man. GP[e]	0.30	CB65	0.25
1299x 1T#	0.37[b]	Guat. GpO30-1A	0.29	B-20#	0.24
MAS:gk	0.36	1520#	0.29	ETO X's	0.22
PR70B602-604	0.36	Cow Corn	0.28	GT-CEW-R58	0.22
Z. Chico (2451)#	0.35[d]	Oloton No. 1#	0.27	Kyle LES[f]	0.22
Tabloncillo X's	0.34	Dial-4 P28	0.26	Strawberry D.	0.20

[a] Single-year determination. [b] 2-year average. [c] 3-year average. [d] 5-year average. [e] Colorado Manfredi GP. [f] Kyle Long Ear Syn.

Table III. Variability of Maysin Levels in Individual Silks of High-Maysin Inbreds and Populations

corn line	type[a]	maysin level[b]	SD	N
CI317B	I	0.427	0.109	12
GT114 (1989)	I	0.362	0.128	12
GT114 (1991)	I	0.538	0.253	20
Akd24	I	0.318	0.074	12
2-043	I	0.306	0.107	12
Ab618	I	0.202	0.053	11
998X 1T#	P	0.448	0.322	12
1299X 1T#	P	0.397	0.246	13
ZC	P	0.297	0.181	21
1520#	P	0.287	0.315	12
PI340856	PI	0.743	0.731	31

[a] I, inbred; P, population; PI, plant introduction. [b] Percent fresh weight, single-year data.

were stored at 0 °C until analysis. For single-silk analyses, the entire silk bundle from a single ear (only one ear per plant was sampled), was placed in a 4-oz jar and covered with 100 mL of MeOH (100%). Samples were warmed to room temperature, and 6 mL of chrysin dissolved in methanol (1.6 mg/mL, recrystallized from amyl alcohol) was added as internal standard. After ultrasonication for 20 min, aliquots of the solution were analyzed by reversed-phase HPLC, as described before (Snook et al., 1989), using a H_2O/MeOH linear gradient from 20% to 80% MeOH in 35 min, a flow rate of 1 mL/min, and detection at 340 nm. Each solvent contained 0.1% H_3PO_4. Most analyses were performed with a used Altex Ultrasphere C18, 5 μm (4.6 × 250 mm, Beckman Instruments, Norcross, GA) column. Additional analyses were made with a Hypersil Phenyl, 5 μm (4.6 × 250 mm, Alltech Associates, Deerfield, IL) column.

Isolation of Flavone Glycosides. All solvents were of analytical reagent grade. Fast atom bombardment mass spectral data (FAB-MS) were obtained in a glycerol matrix, as described before (Snook et al., 1991). ^1H and ^{13}C NMR data were obtained in DMSO-d_6 at either room temperature or 60 °C with Bruker AM250 or AC300 spectrometers.

Maysin. Maysin was obtained from the silks of ZC (Snook et al., 1989). FAB-MS: m/z 577 M + H, 431 M + H − rhamnose.

Apimaysin [2″-*O*-α-L-Rhamnosyl-6-*C*-(6-deoxy-*xylo*-hex-os-4-ulosyl)apigenin]. Silks of NC7 and SC353 were the source

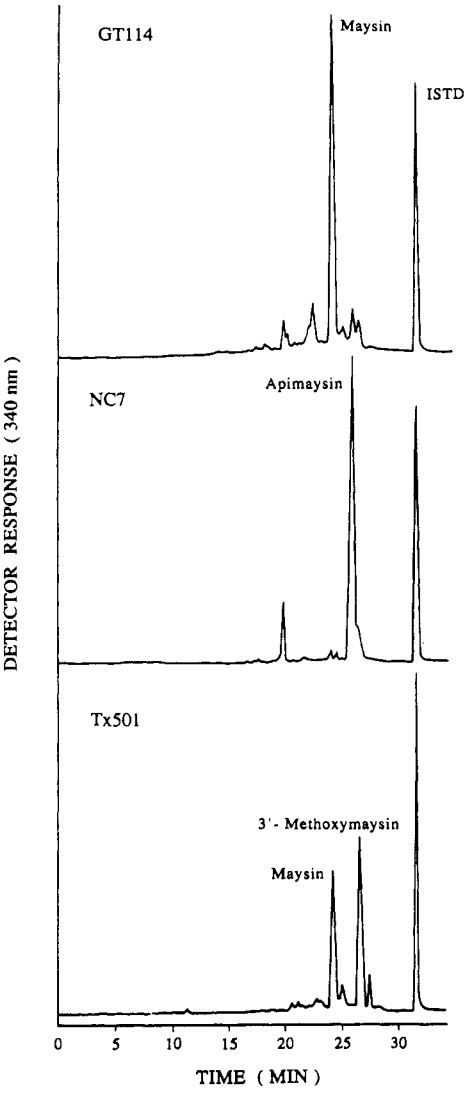

Figure 2. Inbreds with high maysin, apimaysin, and 3′-methoxymaysin levels.

of this compound. A mixture of 162 NC7 silks (1200 g fresh weight) and 102 SC353 silks (486 g) was slurried with 7.3 L of 100% MeOH and filtered, and the MeOH/H_2O solution was concentrated with a rotary evaporator to an aqueous solution of approximately 300 mL. The resulting water/sample mixture was extracted with CH_2Cl_2 (5 × 100 mL), followed by extraction with 1-butanol (3 × 100 mL). HPLC analysis of the aqueous layer showed that a near-quantitative extraction of the flavone glycosides was obtained in the 1-butanol extract. The 1-butanol was evaporated to dryness (a small amount of water, added at the end of the evaporation, facilitated the removal of the last traces of 1-butanol). The residue was dissolved in 40 mL of 40% MeOH/H_2O and submitted to preparative reversed-phase column chromatography. The packing material from a Waters PrepPAK 500 C_{18} cartridge (Millipore Corp., Milford, MA) was repacked into a smaller glass Cheminert LC column (54 × 2.54 cm, Valco Instruments Co., Inc., Houston, TX), washed with MeOH and recycled to 40% MeOH/H_2O. The 1-butanol residue was chromatographed in two batches, by applying half of the sample to the column with a 20-mL loop injection valve and eluting with the following linear solvent program: 40–60% MeOH/H_2O in 400 min; 8-mL fractions collected; column effluent monitored at 340 nm. Combined fractions 58–92 yielded apimaysin in >80% purity. The material from both runs was combined and rechromatographed with a 35–60% MeOH/H_2O solvent program to produce fractions containing apimaysin in >98% purity. Fractions, containing 80–98% pure apimaysin, were combined and chromatographed again on the reversed-phase column. Fractions from this run, containing pure (>98% by HPLC) apimaysin, were combined with those of the previous run and yielded a total

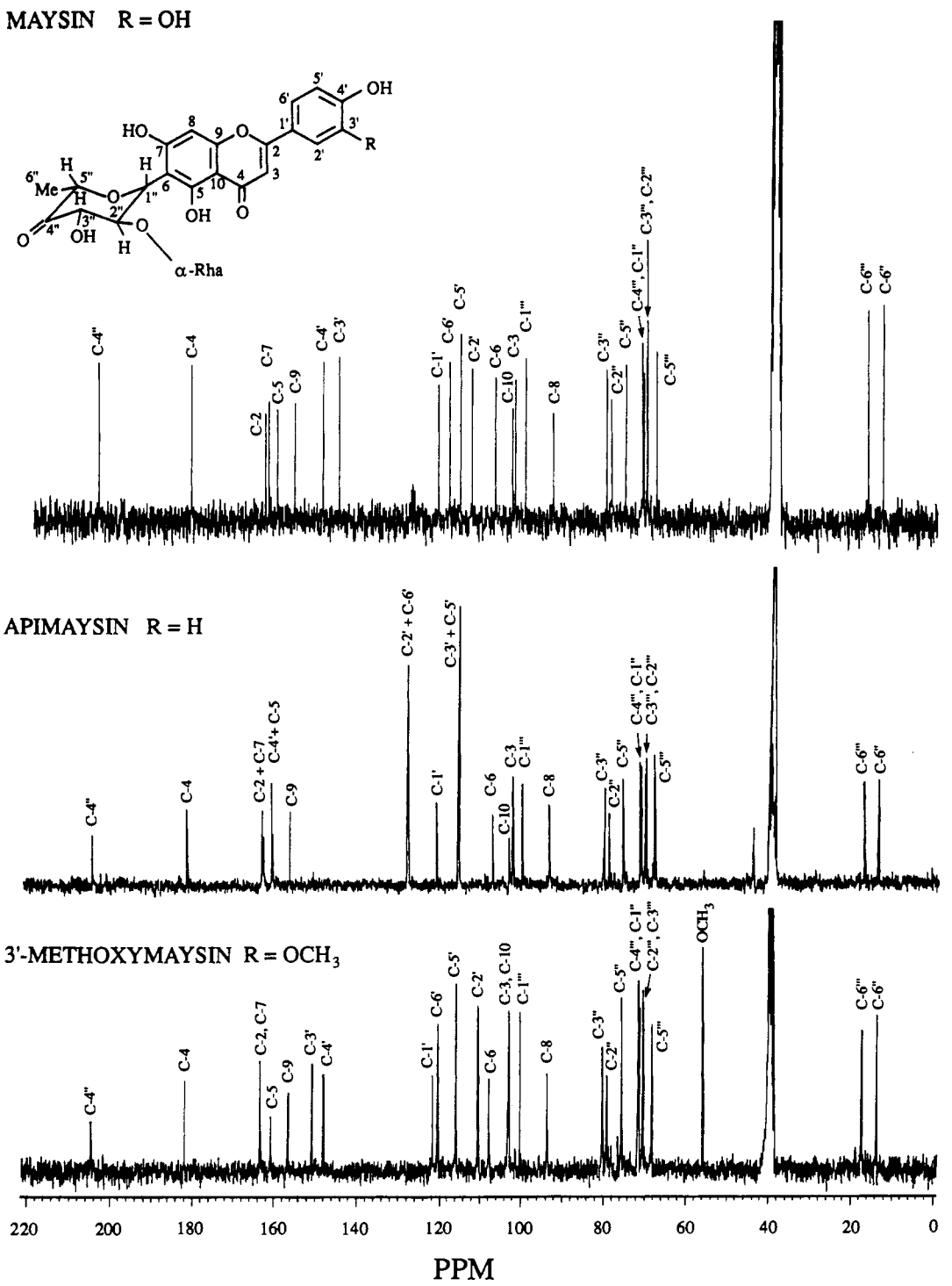

Figure 3. ^{13}C NMR spectra of maysin, apimaysin, and 3′-methoxymaysin (see Table IV).

of 171 mg of isolated apimaysin: FAB-MS *m/z* 561 M + H, 415 M + H − rhamnose; decomposition point 205 °C.

Evaporation of MeOH/H_2O solvents from maysin or apimaysin produced a yellow, glasslike residue, due to tightly bound water. This water was conveniently removed by dissolving the residue in MeOH and adding an equal amount of acetonitrile. Upon evaporation of this solvent, an amorphous yellow powder was obtained.

3′-Methoxymaysin [2-*O*-α-L-Rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)-3′-methoxyluteolin]. Silks (109 silks, 641 g in 3.2 L of MeOH) of Tx501 were extracted with methanol, CH_2Cl_2, and 1-butanol as described above for apimaysin. The 1-butanol residue was dissolved in 500 mL of H_2O and chromatographed on an open tube column (2.54 × 50 cm) packed with 100 g of PrepPAK 500 C_{18} in water. The column was eluted with 0.5 L of H_2O, 1.25 L of 20% MeOH/H_2O to elute maysin, and 2 L of 30% MeOH/H_2O to elute the 3′-methoxymaysin. The crude 3′-methoxymaysin was rechromatographed on the reversed-phase column, described for apimaysin (solvent program—linear gradient from 40% to 60% MeOH/H_2O in 400 min). Fractions 113–118 yielded 50 mg of 3′-methoxymaysin in >97% purity: FAB-MS *m/z* 591 M + H, 445 M + H − rhamnose; decomposition point 176–180 °C with melting at 195 °C.

RESULTS AND DISCUSSION

Flavone analyses were performed by reversed-phase high-performance liquid chromatography on the methanol extracts of the silks of 370 inbreds and 160 populations of corn, selected as representing a broad genetic base. In addition, 81 plant introductions (PIs) and 36 unassigned germplasm sources of corn from the North Central

Table IV. ^{13}C NMR Chemical Shift Assignments of Maysin, Apimaysin, and 3′-Methoxymaysin ([$^2H_6$]Dimethyl Sulfoxide)

carbon assignment[a]	maysin	apimaysin	apigenin[b]	3′-methoxy-maysin	3′-methoxy-luteolin[b]
C-4″	204.1	204.3		204.4	
C-4	181.5	181.6	181.8	181.6	181.8
C-2	163.4	163.3	164.1	163.1 (C-2′ + 7′)	164.2
C-7	162.6	162.9	163.8		163.7
C-5	160.5	160.6	161.1	160.5	161.6
C-9	156.3	156.4	157.3	156.4	157.4
C-4′	149.4	160.9	161.5	147.9	148.0
C-3′	145.5	115.8 (C-3′ + 5′)	116.0 (C-3′ + 5′)	150.7	150.8
C-1′	121.3	121.0	121.3	121.3	121.7
C-6′	118.6	128.1 (C-2′ + 6′)	128.4 (C-2′ + 6′)	120.1	120.4
C-5′	115.9			115.7	115.8
C-2′	113.1			110.4	110.2
C-6	107.5	107.6	98.8	107.6	98.2
C-10	103.3	103.3	103.7	102.9	103.3
C-3	102.6	102.6	102.8	103.2	103.8
C-1‴	100.1	100.1		100.1	
C-8	93.3	93.4	94.0	93.5	94.0
C-3″	80.2	80.2		80.2	
C-2″	79.0	79.0		78.9	
C-5″	75.5	75.6		75.6	
C-4‴	71.5	71.5		71.5	
C-1″	71.1	71.2		71.2	
C-2‴	70.3	70.4		70.4	
C-3‴	70.2	70.2		70.3	
C-5‴	68.0	68.1		68.2	
C-6‴	17.1	17.2		17.3	
C-6″	13.5	13.6		13.7	
OCH_3				55.9	56.0

[a] See Figure 1. [b] Markham and Chari (1982).

Regional PI Station, Ames, IA, were also analyzed. The results of these analyses (Table I) showed that there is a wide range in silk maysin levels, from <0.01% to >0.5% fresh weight. Previously, Waiss (Waiss et al., 1981) showed that 0.15% maysin (w/w) in laboratory bioassay diets reduced corn earworm larval weights by 50%. Wiseman (Wiseman et al., 1992) subsequently showed, in a more detailed study using a variety of different corn lines and crosses, that a highly significant negative relationship ($r = -0.81, P < 0.001$) existed between maysin concentration in fresh silks and weights of corn earworm larvae fed diets containing methanol–silk extracts. Their study indicated that silk maysin concentration of 0.2% reduced larval weights to <50% and that higher maysin levels (>0.4%) reduced larval weights to <70% of controls.

In this study, we found a considerable number of corn inbreds and populations (Table II) with silk maysin levels above the 0.2% fresh weight threshold, considered to be significant for corn earworm antibiosis. Approximately one-fifth of both the inbreds and populations were found to have maysin levels >0.2%. These lines form an important new genetic base for breeding studies to produce agronomically acceptable corn earworm resistant germplasms. Fully one-third of these lines contain silk maysin, which is far greater than ZC, on the basis of the amount of maysin per quantity of silk. Of the PIs evaluated, the silks of PI340856 averaged 0.743% maysin over 3 years. This PI is a popcorn from the Eldredge collection. An unassigned source from Ames, IA, designated Ames 10589 (Junin 107) and collected in Peru, gave 0.914% maysin for a five-silk composite in 1991.

For a number of inbreds and populations, single-silk maysin analyses were made for individual ears of separate plants. The standard deviation data in Table III show that, in general, the populations and PIs exhibited more variability in maysin levels than the inbreds. This trend in variability is well-known for morphological, agronomic, and other chemical characteristics of the groups.

In addition to identifying corn germplasm with high maysin contents, the survey resulted in the discovery of several inbreds with very high levels of flavone glycosides, which were subsequently shown to be previously identified (Elliger et al., 1980a) maysin analogues. HPLC profiles of two of these unique inbreds are given in Figure 2. They are compared to the HPLC profile of GT114, which is typical of a high silk maysin corn. The profile of GT114 is similar to the previously reported high-maysin corn, ZC (Snook et al., 1989). GT114 contained low levels of two flavone glycosides eluting after maysin (retention times 25.8 and 26.4 min). The inbred, NC7, was found to contain high levels of the 25.8-min compound, while Tx501, contained high levels of the 26.4-min compound. These two varieties were convenient sources for the isolation of these two compounds. A new isolation method was developed which was much simpler than that previously reported (Snook et al., 1989) in that it replaced two solvent partitioning steps and one column chromatographic step with a single 1-butanol extraction step. 1-Butanol was found to be particularly effective in extracting the flavone *C*-glycosides from the H_2O/sugar matrix. The compounds were identified by FAB-MS, UV spectroscopy, and ^1H and ^{13}C NMR as 2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)apigenin (which we call apimaysin) from NC7 and 2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)-3′-methoxyluteolin (3′-methoxymaysin) from Tx501.

The ^1H NMR spectra of maysin, apimaysin, and 3′-methoxymaysin agreed with those reported by Elliger (Elliger et al., 1980a). The ^{13}C NMR spectra of maysin, apimaysin, and 3′-methoxymaysin (the latter two presented for the first time) are given in Figure 3, while chemical shift assignments of these and related flavones are given in Table IV. In addition to confirming the aglycon portion of the molecule, the ^{13}C spectra proved that apimaysin and 3′-methoxymaysin both retained the 2″-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl) sugar structure. Two-dimensional ^1H–^{13}C chemical shift correlation (HETCOR) of maysin allowed unequivocal assignments of the sugar ^{13}C resonances, which were only tentatively assigned in our previous work (Snook et al.,

Table V. Inbreds and Populations with High Silk Apimaysin and 3′-Methoxymaysin (Percent Fresh Weight)

	maysin	apimaysin	3′-methoxymaysin
High-Apimaysin Lines			
inbreds			
NC7[a]	0.050	0.614	0.159
Sa4 (W)[a]	0.034	0.205	0.046
SC353[a]	0.402	0.219	0.001
Mp496	0.012	0.083	0.000
populations			
3146x 1T#[a]	0.125	0.280	0.108
Mexican unknown #1	0.200	0.049	0.028
1520#[a]	0.235	0.042	0.042
Puerto Rico #[a]	0.062	0.032	0.016
High-3′-Methoxymaysin Lines			
inbreds			
9-201	0.197	0.000	0.297
SC144	0.179	0.000	0.293
CI64[a]	0.380	0.034	0.266
Tx501[a]	0.169	0.001	0.193
NC7[a]	0.050	0.614	0.159
Mp311	0.249	0.034	0.156
GE80	0.847	0.018	0.154
Mp307	0.039	0.000	0.122
T206	0.031	0.000	0.114
GE76	0.060	0.000	0.110
SC249A	0.219	0.000	0.105
populations			
Kyle Late Syn	0.299	0.001	0.155
998x 1T#[a]	0.454	0.006	0.132
Oloton No. 1#[a]	0.253	0.006	0.109
3146x 1T#[a]	0.125	0.280	0.108
Dial-5 P43	0.262	0.000	0.102
Oax Comp Grp.	0.565	0.008	0.100

[a] 2-year average.

1989). The ^{13}C chemical shifts of the aglycon portion of 3′-methoxymaysin were in excellent agreement with those of 3′-methoxyluteolin but not of 4′-methoxyluteolin (Markham and Chari, 1982), confirming the assigned structure.

As mentioned under Materials and Methods, most analyses were performed with an aged Altex Ultrasphere C_{18} column. New Ultrasphere C_{18} columns failed to separate apimaysin from 3′-methoxymaysin, although they were adequate for maysin analysis. Of several other columns tested, only a Hypersil Phenyl column sufficiently resolved apimaysin and 3′-methoxymaysin.

Although most corn lines with high maysin levels also contained small amounts of either apimaysin, 3′-methoxymaysin, or both compounds, very few lines contained large amounts of these flavones. Elliger et al. previously isolated apimaysin and 3′-methoxymaysin from ZC, in which they occur in minor amounts (Elliger et al., 1980a). Our analysis of ZC showed apimaysin and 3′-methoxymaysin to be present in only 0.019% and 0.045% fresh weight, while maysin was at the 0.35% level (averaged over 4 years). Table V lists germplasm sources that were found to contain high levels of apimaysin and 3′-methoxymaysin. Only one line, the inbred NC7, was found to contain appreciable levels of apimaysin. It is unique in the quantity of this flavone it produces (0.614% fresh weight), along with only a minor amount of maysin. SC353 is another good source of apimaysin. Silks of the population 3146x 1T# had 0.280% apimaysin but also contained about equal levels of maysin and 3′-methoxymaysin. Apimaysin levels in all other populations were less than one-fifth of that in 3146x 1T#.

Inbred corn sources of 3′-methoxymaysin were more numerous than those for apimaysin; however, 3′-methoxymaysin occurred in the silks at levels approaching only 0.3% fresh weight (Table V). Lines such as Tx501 and SC144 are the best sources, since they have little or no apimaysin which is difficult to separate from 3′-methoxymaysin. Populations were not found to be particularly good sources of this compound.

The predisposition of NC7 to produce apimaysin instead of maysin makes it a prime candidate to study the 3′-hydroxylation step in the synthesis of flavones of corn, as the enzyme to accomplish this is apparently severely inhibited. The enzyme could be completely absent in NC7, because the very small LC peak eluting at maysin's retention time could be another flavone, eluting at the same time. Activation of this enzyme and others involved in the biosynthetic pathway to flavones may make it possible to increase maysin production to levels adequate for corn earworm resistance in lines already acceptable for their agronomic properties.

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