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Expression of a Maize *Myb* Transcription Factor Driven by a Putative Silk-Specific Promoter Significantly Enhances Resistance to *Helicoverpa zea* in Transgenic Maize

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Hi II maize (*Zea mays*) plants were engineered to express maize *p1* cDNA, a *Myb* transcription factor, controlled by a putative silk specific promoter, for secondary metabolite production and corn earworm resistance. Transgene expression did not enhance silk color, but about half of the transformed plant silks displayed browning when cut, which indicated the presence of *p1*-produced secondary metabolites. Levels of maysin, a secondary metabolite with insect toxicity, were highest in newly emerged browning silks. The insect resistance of transgenic silks was also highest at emergence, regardless of maysin levels, which suggests that other unidentified *p1*-induced molecules likely contributed to larval mortality. Mean survivor weights of corn earworm larvae fed mature browning transgenic silks were significantly lower than weights of those fed mature nonbrowning transgenic silks. Some transgenic pericarps browned with drying and contained similar molecules found in pericarps expressing a dominant *p1* allele, suggesting that the promoter may not be silk-specific.

KEYWORDS: ***Helicoverpa zea*; maysin; insect resistance; silk; transgenic**

INTRODUCTION

Insect herbivores cause crop losses through physical damage but can also contribute to the colonization of plants by fungi, some which produce toxins deleterious to livestock and humans (*1*). Enhancing plant resistance to insect feeding is an important strategy of crop management (*2*). The development of transgenic plants with insect resistance compounds has enabled valuable crops to be armed with new sources of durable resistance unavailable through breeding (*3*). Most of the time, the insect resistance compounds are produced constitutively throughout the plant, but in some cases, it may be preferable to express the toxin in a tissue-specific or -inducible manner (*3*). Phloem-specific promoters expressing lectins have been successfully used to counter sap-sucking insects in transgenic tobacco, rice, and Indian mustard (*4*−*7*). Modified toxins from *Bacillus thuringiensis* have been expressed in pollen, pith, and only green tissues for effective pest control in transgenic rice and tobacco (*8*−*11*). Pollen-, green tissue-, and root-specific promoters have been utilized in maize for insect control as well (*12*, *13*).

Corn earworm and fall armyworm larvae can enter ears through protruding silks. To reduce corn earworm and fall armyworm damage and to avoid consumer concerns, it would be advantageous to develop maize lines that produce insect-resistant molecules only in the silks. In the course of developing a method for hybrid seed production, a putative silk-specific gene (pSH64), including 5′- and 3′-regulatory regions, was isolated from a Mo17 genomic library (*14*). Expression of the uncharacterized 615 bp (base pair) open reading frame was found in silks but not in tassel, leaf, or root tissues (*14*). More recently, a silk-expressed gene, *Zea mays* glycine-rich protein 5 (*zmgrp5*), was isolated from two maize inbred lines (*15*). Detailed molecular characterization of *zmgrp5* in silks indicated that the cell wall-localized protein was expressed in silk hair, pollen tube-transmitting tissue elements, and cells of the vascular bundle (*15*). The putative protein-coding regions of pSH64 and *zmgrp5* are 97% identical at the nucleotide level except that pSH64 appears to have a 123 bp intron while the two protein sequences are 96% identical. Thus, it is likely that pSH64 and *zmgrp5* encode the same protein in silk tissue.

The maize *p1* gene has been well-characterized at the genetic and molecular level. *p1* encodes a *Myb*-related transcriptional activator that regulates production of 3-deoxy anthocyanins, phlobaphenes (polymers of flavan-4-ols), and C-glycosyl flavones (*16*). Many of the biosynthetic steps regulated by *p1* are also utilized for anthocyanin biosynthesis (*16*). The ears differentially accumulate phlobaphenes depending on the allele: *P1-wr* (white pericarp/red cob), *P1-rr* (red pericarp/red cob), *P1-rw* (red pericarp/white cob), and *p1-ww* (white pericarp/white cob). In silks, *p1* directs the biosynthesis of C-glycosyl flavones including isovitexin, isoscoparin, maysin, apimaysin, and 3-methyoxymaysin (*17*). Maysin has been found to inhibit the growth of corn earworm larvae (*18*). A previous study used

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the *P1-rr* or *P1-wr* promoter to express *p1* cDNA in *p1-ww* germplasm and found increased levels of maysin in silks with varying levels and distribution of pigmentation in pericarps and other tissues but did not examine the activity against insects (*19*). It would be useful to determine if *p1*-mediated maysin production in silks could be uncoupled from colored pericarps by silk-specific *p1* gene expression and increase corn earworm resistance. To test this possibility, a gene expression vector was constructed in which the *p1* cDNA was inserted in between the putative silk-specific promoter and terminator from pSH64.

MATERIALS AND METHODS

DNA Plasmid Constructs. The silk-specific promoter from pSH64 (from U.S. Patent 6,392,123 deposited at the Agricultural Research Service Patent Culture Collection in Peoria, IL, and assigned accession number NRRL B-21920) was amplified by *PfuUltra* DNA polymerase (Stratagene, La Jolla, CA) using the forward primer CGC CGA TAT CGG ATC CTT AGA TCT TTA GG, the reverse primer TCC CCC GGG TTT CTT GCT GTA GTC CTC, and Perfect Match polymerase chain reaction (PCR) enhancer (0.5 μL/50 μL PCR reaction, Stratagene) according to the manufacturer's recommendations and the following thermal cycling conditions (95 °C for 2 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min, extending 10 min at 72 °C, and holding at 2 °C) in a MJ Research (Waltham, MA) PTC-150 Minicycler. The amplified promoter was cut with BamH I and Sma I and cloned into pBluescript KS+. The maize *p1* cDNA was cloned downstream of the silk-specific promoter as a blunt-end XhoI fragment. The silk-specific terminator from pSH64 was amplified by *PfuUltra* DNA polymerase using the forward primer CTC GAG ATC ATG GCG GTG GTG GTG CAC, the reverse primer GGT ACC TGG TGC CCA GCA GAT AGAA, Perfect Match PCR enhancer (as above) according to the manufacturer's recommendations, and the following thermal conditions (95 °C for 2 min, 30 cycles of 95 °C for 30 s, 66.5 °C for 30 s, 72 °C for 4 min, extending 10 min at 72 °C, and holding at 10 °C) in a MJ Research PTC-200 thermal cycler. The amplified terminator was cloned downstream of the maize *p1* cDNA as an Xho I−Kpn I fragment, and the final construct was named silk-P-silk.

Maize Transformation. Immature Hi II maize embryos were bombarded with silk-P-silk plasmid and a selectable marker gene at the Iowa State Plant Transformation Facility (*20*). Transgenic plants were grown in a greenhouse with the target temperature conditions of 25 °C and a 14:10 light/dark photoperiod maintained by supplemental lights. Transgenic silks were pollinated with B73 pollen after the initial cutting (see below). After removal of 3 week old silks and husks, the cobs (covered with their ear bags) were allowed to dry in the greenhouse.

Bioassays. The *Helicoverpa zea* were reared at 27 ± 1 °C, 40 ± 10% relative humidity, and a 14:10 light:dark photoperiod on a pinto bean-based diet as previously described (*21*−*23*). Newly emerged silks about 3−6 cm in length were cut, and some (sufficient for ad libitum feeding) were placed in 5 cm diameter Petri plates with tight-fitting lids (Falcon 351006, Becton Dickinson Labware, Franklin Lakes, NJ) while another portion was frozen at −20 °C for metabolite analysis. Ten corn earworm neonate larvae were added to each sample, and the dishes were held in the dark at 27 ± 1 °C and 40 ± 10% relative humidity for 48 h. Larva mortality was scored at 24 and 48 h. Silks were scored for browning when larvae mortality was checked at 24 h. Silks were also removed from nonpollinated and pollinated ears (at milk stage) about 3 weeks after the first cutting and assayed for insect activity as just described while another portion of silks was frozen as above. Only 3 week old silks below the husk were used for bioassays since most of the silk tissue outside the husk was dry. Larvae surviving the 48 h bioassay were frozen and subsequently weighed to 0.01 mg using an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus, OH).

Silk Metabolite Analysis. Frozen silks were weighed and submerged in methanol at least overnight at 4 °C. The total methanol volume was measured with a 250 μL syringe and passed through a 0.45 μm syringe filter (4 mm diameter, Alltech Associates Inc., Deerfield, IL). Maysin analysis was done by reversed-phase high-pressure liquid chromatography (HPLC) (*24*) except that a different column and solvent system were used. A portion of the sample was injected on to a reversed-phase Inertsil 5 μm ODS-3 HPLC column (250 mm × 4.6 mm, Metachem Technologies Inc., Torrance, CA) in an Agilent Technologies (model 1100) HPLC system with the detector set to monitor effluent at 340 nm. The mobile phases were 0.025% trifluoroacetic acid (A) and acetonitrile containing 0.025% trifluoroacetic acid (B) running at a flow rate of 1 mL/min with the following timetable: 0 min, 15% B; 30 min, 65% B; 40 min, 100% B; 45 min, 100% B; 48 min, 15% B; and 50 min, 15% B. Chromatograms were integrated using the Chemstation for LC 3D (Rev. A.10.02) and compared to a maysin standard (from M. E. Snook) ranging from 0.365 to 16 μg.

Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (LC-ESI-MS) Analysis. Samples were run on a Finnigan-Thermoquest LC-MS system [Surveyor autoinjector, quaternary HPLC pump, photodiode array (PDA) detector, and LCQ Deca XP plus ion-trap mass spectrometer] (San Jose, CA) all running under the Xcaliber 1.3 software system. The MS was run with the ESI probe in the positive mode. The column was a 3 mm × 150 mm Inertsil reverse phase C-18, ODS 3, 3 μm column (Varian, Torrance, CA) with a Metaguard guard column. The source inlet temperature was set at 220 °C, and the sheath gas rate was set at 90 arbitrary units. The solvent systems were (A) water with 0.25% acetic acid and (B) methanol with 0.25% acetic acid. The column was equilibrated with 2% B at a flow rate of 0.3 mL min^{-1}. After injection, the column was held at the initial conditions for 2 min, then developed with a linear gradient to 40% B over 23 min, and then to 50% B over the next 10 min. The column effluent was monitored at 280 and 310 nm in the PDA detector. The software package was set to collect mass data between 150 and 2000 atomic mass units. The MS was optimized by infusing pure maysin dissolved in methanol using the autotune feature of the software. The MS was tuned on *m*/*z* 577 $[M + H]^+$. The identity of maysin in two transgenic silk extracts was confirmed by comparison with a maysin standard. Both the standard and the silk extract maysin eluted as a peak with two shoulders. The peak had masses of 577 (the mass of maysin is 576.14) and 595 and appeared to be a mix of closely related sugar conjugates of luteolin based on further examination of the standard with nuclear magnetic resonance (NMR) at Peoria.

Pericarp Metabolite Analysis. *Z. mays* seeds genotyped *P1-rr* and *P1-rw* were obtained from the Maize Genetics Cooperation Stock Center in Urbana, IL. All seeds were soaked in water for a number of days until germination, and then, pericarps were removed. The pericarps were frozen in liquid nitrogen and ground in a mortar and pestle. The pericarps were further macerated after the addition of methanol containing 0.1 N HCl. The pericarp acidic methanol extracts were stored at least overnight at 4 °C. The extracts were evaporated to reduce the volume and then filtered before being injected into the HPLC. Sample 7E was further purified with a Waters Sep-Pak-Plus tC18 cartridge (Milford, MA) to remove some contaminants. The same HPLC system and column described above were used while the detector monitored effluent at 520 nm. The mobile phases were 1% formic acid (A) and acetonitrile (B) running at a flow rate of 1 mL/min with the following timetable: 0 min, 0% B; 35 min, 39% B.

Statistics. Analysis of variance (ANOVA) and correlation analysis (REG procedure) were performed using SAS 9.1 for Windows, 2003 (SAS Institute Inc., Cary, NC).

RESULTS

Secondary Metabolite Production in Transgenic Silks. Fifty-six percent of the transgenic ears developed silks that displayed a browning reaction when cut. LC-ESI-MS analysis determined that transgenic browning silks contained maysin (see the Materials and Methods). Measurement of maysin levels by HPLC methods in transgenic silk extracts indicated that browning silks contained significantly more maysin than nonbrowning silks (**Table 1**). B73, which had browning silks and is *P1-wr*, had less maysin in its newly emergent silks than the transgenic browning silks, but the mean values were not significantly

Table 1. Maysin Levels in Various Silk Tissues[a]

Newly Emergent Silks	
transgenic browning	0.36 ± 0.042 (22) a
transgenic nonbrowning	0.0034 ± 0.0024 (12) b
B73	0.19 ± 0.085 (4) a
Hi II	0.0039 ± 0.0022 (6) b
3 Week Old Silks (outside Husk)	
transgenic browning	0.12 ± 0.019 (15) a
transgenic nonbrowning	0.0082 ± 0.0020 (7) b
B73	0.13 ± 0.028 (3) a
Hi II	0.0031 ± 0.0011 (4) b
3 Week Old Silks (under Husk)	
transgenic browning	0.11 ± 0.024 (21) ab
transgenic nonbrowning	0.0026 ± 0.0005 (11) cd
B73	0.041 ± 0.021 (4) ebf
Hi II	0.0012 ± 0.00072 (5) gdf

[a] All values are the mean % fresh weights with the standard error. The number of samples analyzed is in parentheses. Mean values in rows followed by a different letter are statistically significant at $P < 0.05$ by ANOVA.

Table 2. Insect Mortality in Bioassays Using Various Silk Tissues[a]

	day 1	day 2
	newly emergent silks	
transgenic browning	60 ± 3.2% (85) a	87 ± 2.1% (85) a
transgenic nonbrowning	54 ± 3.8% (72) a	81 ± 3.1% (72) a
B73	49 ± 13% (6) ab	82 ± 5.5% (6) ab
Hi II	26 ± 5.8% (7) b	59 ± 12% (7) b
	3 week old silks	
transgenic browning	4.7 ± 1.4% (87) a	5.3 ± 1.6% (87) a
transgenic nonbrowning	3.0 ± 0.72% (71) a	3.4 ± 1.0% (71) a
B73	0.0 ± 0.0% (6) a	1.0 ± 1.0% (6) a
Hi II	0.0 ± 0.0% (7) a	0.0 ± 0.0% (7) a

[a] The number of bioassays analyzed is in parentheses. Mean values ± standard errors in rows followed by a different letter are statistically significant at $P < 0.05$ by ANOVA.

different as compared to transgenic browning silks at $P < 0.05$. Hi II newly emergent silks contained about the same maysin as nonbrowning transgenic silks. About 3 weeks after the initial removal of silks, ears were removed and silk samples above and below the husks were frozen for future analysis. At this time, most of the successfully pollinated cobs contained kernels at the milk stage. Fifty-five percent of the 3 week old transgenic ears contained silks (under the husk) whose cut ends displayed a browning reaction. The 3 week old transgenic browning silks, those both under and out of the husk, contained significantly more maysin than the respective nonbrowning silks (**Table 1**). Maysin levels in 3 week old transgenic browning silks outside the husk (0.12%) were significantly lower than newly emergent transgenic browning silks (0.36%, ANOVA, $P < 0.0001$). Maysin levels in B73 silks (newly emergent vs outside of husk) were slightly lower after 3 weeks but not significantly. Maysin levels were low in all transgenic nonbrowning silks and in Hi II nontransgenic controls regardless of position after 3 weeks.

Insect Resistance of Transgenic Silks. Larvae feeding on browning and nonbrowning newly emergent transgenic silks exhibited higher levels of mean mortality than those feeding on Hi II newly emergent silks on both days of the bioassay (**Table 2**). However, there was no significant difference at $P < 0.05$ between the mortality rates of larvae feeding on newly emergent transgenic browning and nonbrowning silks on both days of the bioassay. Larvae feeding on B73 newly emergent silks displayed mean mortality levels not significantly different at $P < 0.05$ to those feeding on the transgenic silks and Hi II silks on both days of the bioassay. Mean mortality of larvae feeding on all types of 3 week old silks was dramatically lower (<10%) than mean mortality rates of larvae feeding on newly emergent silks. Mean mortality rates on the different 3 week old silk tissues were not significantly different at $P < 0.05$ on both days of the bioassay. Surviving larvae feeding on the 3 week old silk tissues were weighed after the bioassay (**Table 3**). Larvae that had eaten B73 silks were significantly smaller than all others, and larvae that had eaten Hi II silks were significantly larger than all others. The larvae feeding on the transgenic silks were intermediate in size, but those that ate the transgenic browning silks were 13% smaller (significant at $P < 0.05$) than those that ate the transgenic nonbrowning silks. Larval survivor weights were significantly negatively correlated to maysin levels in transgenic browning silks but not transgenic nonbrowning silks. Combining the data of both browning and nonbrowning transgenic silk larva survivors also results in a significant negative correlation between larval weight and maysin levels. The larval weights of survivors that fed on B73 and Hi II silks were significantly negatively ($R = -0.41$) and positively ($R = 0.34$) correlated to maysin levels, respectively.

Table 3. Mean Weights of Survivors Feeding on 3 Week Old under-the-Husk Silks[a]

silk tissue	mean larva weight in mg (N)	correlation coefficient[b] (P)
transgenic browning	0.34 ± 0.0067 (673) a	− 0.29 (0.0002)
		–0.25 (<0.0001)[c]
transgenic nonbrowning	0.39 ± 0.0073 (539) b	–0.035 (0.75)
B73	0.27 ± 0.019 (62) c	–0.41 (0.012)
Hi II	0.45 ± 0.03 (73) d	0.34 (0.022)

[a] Mean larva weights (±standard error) in rows followed by a different letter are statistically different at $P < 0.05$ by ANOVA. [b] Correlation of larva weight to maysin levels. [c] Correlation of weights of larvae feeding on transgenic browning and nonbrowning silks to maysin levels.

***p1* Expression in Transgenic Pericarps.** After a few days of drying, some transgenic kernels displayed a browning reaction (**Figure 1A**). With further drying, 69% of the transgenic ears producing kernels had pericarps showing a light brown to rust color that closely resembled the color of *P1-rw* pericarps (**Figure 1B**). The other transgenic ears had light yellow kernels that looked like the B73 X Hi II kernels (**Figure 1B**). In 20 of 22 ears, those lines with brownish transgenic pericarps had been rated for browning silks at emergence or at 3 weeks after initial cutting while in nine of 10 ear lines, light yellow transgenic pericarps had been rated nonbrowning silks at emergence or at 3 weeks after initial cutting. Selfed B73 pericarps were bright yellow (not shown). HPLC chromatographs of acidic methanol extracts from transgenic brown pericarps were similar to those from *P1-rr* and *P1-rw* pericarps (**Figure 2**). The largest peak was present in all four samples at a similar retention time (20.7−21.0 min). A smaller peak at 18.2−18.6 min was also present in all four samples. Some peaks after 21 min were present in the *P1-rr* and *P1-rw* pericarp extracts but not in the transgenic pericarp extracts.

DISCUSSION

Transforming the silk-P-silk gene construct into Hi II germplasm resulted in over 50% of the transgenic ears containing silks that displayed a browning reaction, characteristic of *p1*-induced secondary metabolite production (*18*), at two different stages of silk development. Levels of maysin, a *p1*-induced secondary metabolite, were highest in newly emerged browning transgenic silks. A large survey of 497 inbred lines

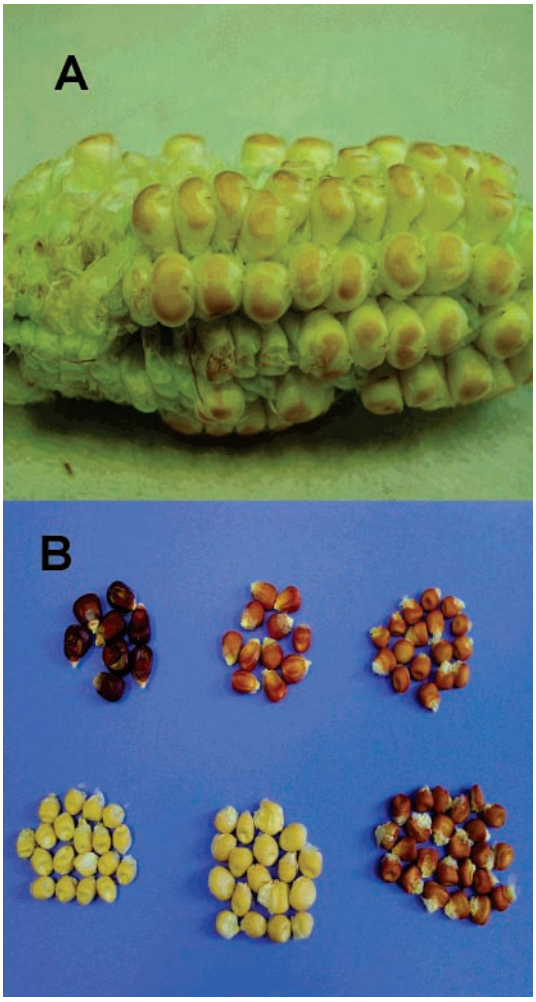

Figure 1. Phenotypes of transgenic kernels. (**A**) Transgenic cob a few days after husk removal. (**B**) Fully dried kernels: Top row, left to right, *P1-rr*, *P1-rw*, and transgenic line 28A × B73; bottom row, left to right, Hi II × B73, transgenic line 15A × B73, and transgenic line 7E × B73.

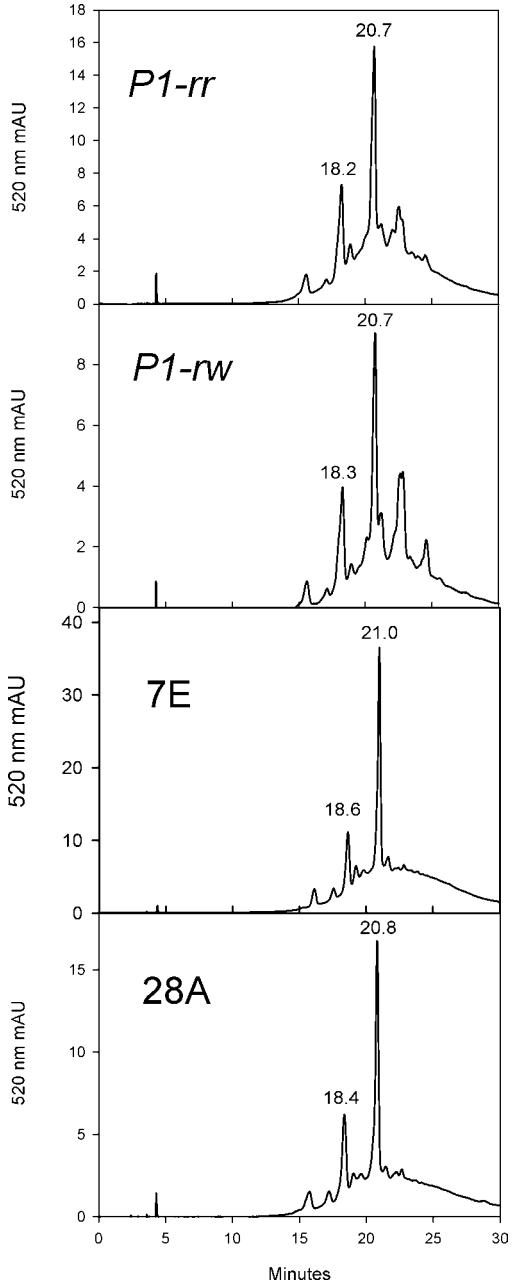

Figure 2. HPLC chromatographs of pericarp extracts. *P1-rr* and *P1-rw* carry a dominant *p1* allele while the two transgenic lines (7E and 28A) were the progeny pericarps of silks crossed with B73 pollen. Scaling of ordinate axis is not uniform to demonstrate similarity of peaks. Numbers above peaks are retention times.

and 295 wild populations found one-fifth with newly emerged silk maysin levels >0.2% FW (*25*). The mean maysin value for transgenic newly emerged browning silks fits into this upper tier of maysin production, suggesting that expression from the putative silk-specific promoter is more than adequate for secondary metabolite production at the early stages of silk emergence. Because the maysin levels in newly emerged transgenic browning silks and B73 silks are statistically equivalent, the silk-P-silk gene construct directs *p1*-induced metabolite production at levels comparable to a functional *P1-wr* allele. However, unlike the *P1-wr* allele, only a few transgenic cobs with light pigmentation were observed. Maysin levels in transgenic nonbrowning silks were dramatically lower, paralleling levels in Hi II silks. Browning was never observed in Hi II cut silks of any age. Hi II is phenotypically *p1-ww* and has served as a transformation host for a number of *p1* transgenes (*19*, *26*).

Maysin levels in the transgenic silks declined between the initial emergence and the final harvest. A reduction of maysin levels in silks over time has been documented for the high-maysin Zapalote Chico cultivar (*27*). Maysin levels in silks also generally decline 1 or 2 days after the first cutting (*28*). Thus, both cutting and development lead to reductions in silk maysin production. The majority of the 3 week old silks were previously cut after emergence, so it is not possible to discuss the developmental aspects of the silk promoter.

A large proportion of transgenic silks, both newly emergent and 3 weeks old, did not display a visible browning reaction. One possible explanation is that the *p1* gene was silenced due to overexpression and masyin biosynthesis was not induced. However, RT-PCR analysis of total RNA indicated that *p1* mRNA was present in two of three newly emergent nonbrowning transgenic silk lines analyzed (data not shown). There is evidence showing that *P1* transcription factor regulates its target genes in an additive manner (*29*). Further experimentation is needed to identify the insect resistance factors in the nonbrowning transgenic silks.

Larva mortality was low (<10%) in 3 week old silks for both Hi II control and all transgenic silks. Even though B73 and

transgenic browning 3 week old under-the-husk silks had statistically equivalent levels of maysin, larvae survivors feeding on B73 3 week old silks weighed significantly less than those feeding on transgenic browning silks. There may be additional insect resistance molecules in B73 silks that slow the growth of corn earworm larvae. Interestingly, the larvae survivors feeding on the 3 week old transgenic nonbrowning under-the-husk silks were significantly smaller than those survivors feeding on the 3 week old Hi II under-the-husk silks. As discussed above, the gene construct may have induced insect resistance molecules besides maysin in the 3 week old nonbrowning silks that contributed to slowing larvae growth. It is not clear why larval weight is positively correlated to maysin levels in Hi II under-the-husk silks.

It was surprising to find a large percentage of transgenic pericarps phenotypically similar to *P1-rw* kernels. In the patent, the putative silk-specific cDNA clone pSH64 (which was used to clone the putative silk-specific promoter and terminator for this study) was not expressed in tassels, leaves, or roots. However, pericarp mRNA was not included in the gene expression experiment (*14*). More recently, *zmgrp5* expression was not detected in mRNA or protein extracts from kernel-rachis tissue of the inbred lines CO325 and CO387 (*15*). The putative promoter region from pSH64 is 3790 bp while 1911 bp of the *zmgrp5* promoter was sequenced. A 213 bp segment directly upstream of the translation start site of pSH64 and *zmgrp5* is 93% identical, while region 1368−1755 of the *zmgrp5* promoter is 93% identical to a region in the pSH64 promoter. However, the first 1157 bp of the *zmgrp5* promoter shares no homology with any region of the pSH64 promoter. Thus, it is possible that the similar, but not identical, promoters of pSH64 and *zmgrp5* drive expression of the same glycine-rich protein in silks. More experiments are needed to determine if the promoter of pSH64 drives expression of the glycine-rich protein in pericarp. Maize silks are outgrowths of two anterior carpels, while the pericarp is derived from the two anterior carpels and the posterior carpel (*30*). Perhaps pSH64 is actually a carpel-specific gene that is expressed in both silks and pericarps.

Hi II embryos transformed by particle bombardment with intact and modified *P1-wr* alleles resulted in T_0 plants that produced only red pericarps (*26*). While a single intact *P1-wr* allele (6.2 kb of upstream regulatory sequence plus *P1-wr* cDNA) was transformed into Hi II, this gene construct differed dramatically from the actual *P1-wr* locus, which contains six head to tail tandem repeats of 6.3 kb of upstream sequence and 6.3 kb of genic sequence (*31*). This peculiar arrangement of *P1-wr* may contribute to its expression only in cob tissue (*31*) that was lost when transformed with only 6.2 kb of *P1-wr* upstream sequence and the *P1-wr* cDNA. It may be possible that unknown structural features or sequences upstream of the pSH64 promoter are necessary to prevent *p1* expression in pericarp tissue.

Tissue-specific expression of insect resistance molecules may be advantageous in cases where humans and livestock cannot tolerate high levels of the insect resistance molecules in grains. These results indicate that the silk-specific promoter sufficiently expresses the *p1* gene at a level comparable to the *P1-wr* allele since maysin levels were similar in B73 and transgenic maysin-producing silks at two different points in silk development. However, unlike the *P1-wr* allele, the "silk-specific" promoter expressed *p1*-driven metabolites in transgenic pericarps. Because this result may be due to a deficiency in the promoter sequence or to an unidentified epigenetic mechanism, further studies are needed. Alternatively, there may be some utility in expressing a gene of interest in both silks and pericarps.

ABBREVIATIONS USED

bp, base pairs; ESI, electrospray ionization; HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography−mass spectrometer; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PDA, photodiode array.

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