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ORIGINAL PAPER

Quantitative trait loci for maysin synthesis in maize (*Zea mays* L.) lines selected for high silk maysin content

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Abstract Maysin is a naturally occurring C-glycosyl flavone found in maize (*Zea mays* L.) silk tissue that confers resistance to corn earworm (*Helicoverpa zea*, Boddie). Recently, two new maize populations were derived for high silk maysin. The two populations were named the exotic populations of maize (EPM) and the southern inbreds of maize (SIM). Quantitative trait locus (QTL) analysis was employed to determine which loci were responsible for elevated maysin levels in inbred lines derived from the EPM and SIM populations. The candidate genes consistent with QTL position included the *p* (*pericarp color*), *c2* (*colorless2*), *whp1* (*white pollen1*) and *in1* (*intensifier1*) loci. The role of these loci in controlling high maysin levels in silks was tested by expression analysis and use of the loci as genetic markers onto the QTL populations. These studies support *p*, *c2* and *whp1*, but not *in1*, as loci controlling maysin. Through this study, we determined that the *p* locus regulates *whp1* transcription and that increased maysin in these inbred lines was primarily due to alleles at both structural and regulatory loci promoting increased flux through the flavone pathway by increasing chalcone synthase activity.

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Introduction

Maysin is a naturally occurring C-glycosyl flavone in maize silks which confers resistance to corn earworm (*Helicoverpa zea*, Boddie) (CEW) (Waiss et al. 1979; Elliger et al. 1980; Rector et al. 2002). Previous QTL analyses of maysin content have generally detected a small number of main-effect QTL that explain a large proportion of the phenotypic variance. One of the recurring conclusions is that transcriptional regulators play a key role in controlling variation in maysin content (Byrne et al. 1996b, 1998; McMullen et al. 1998).

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The principle regulatory locus of the flavone pathway is the *p (pericarp color)* locus, which encodes duplicate *myb*-like transcription regulators, *p1* (*pericarp color1*) and *p2* (*pericarp color2*). In F_2 populations generated from one parent with a functional *p* allele and one parent with a nonfunctional *p* allele, the majority of the phenotypic variation in maysin accumulation is explained by the *p* locus (McMullen et al. 1998). The *p* locus confers an additive phenotypic effect because it regulates transcription of mRNA for rate limiting enzymes involved in maysin synthesis (Bruce et al. 2000; McMullen et al. 1998).

Genetic variation in structural genes has also been identified as the basis of QTL for the production of maysin. Chalcone synthase (CHS) is an important enzyme for the flavonoid pathway, as it catalyzes the first committed step (Harborne 1988). In maize, *c2* (*colorless2*) and *whp1* (*white pollen1*) are duplicate loci known to encode CHS and functional alleles for CHS are required for maysin synthesis (Szalma et al. 2002). The *intensifier* (*in1*) locus has been reported to regulate *whp1* expression in aleurone tissue (Coe et al. 1998; Burr et al. 1996; Franken et al. 1991). Although previous studies have shown that *p* regulates *c2* (Bruce et al. 2000; Bushman et al. 2002), the regulation of *whp1* in silk tissue is unknown. With the exception of the *p* locus, no regulatory gene has been tested for effects on maysin content in maize silks.

Genes involved in the nonflavone branches of the flavonoid pathway can also affect maysin accumulation. For example, with loss of function at the *a1* (*anthocyaninless1*) locus, intermediates are shunted from the 3-deoxyanthocyanin pathway to flavone synthesis, increasing maysin accumulation (McMullen et al. 2001). The *rem1* (*recessive enhancer of maysin1*) locus causes similar phenotypic effects but its function is not known (Byrne et al. 1996b).

All of these studies were conducted on standard maize inbred lines. Would the same array of QTLs determine maysin synthesis in lines specifically selected for very high maysin content in maize silks? Widstrom and Snook (2001) used recurrent selection to develop two maize populations for increased maysin content in silks. Germplasm sources for the populations were chosen specifically for their maysin production. Exotic landraces and inbreds were used to derive the "exotic population of maize" (EPM) population and six southern US inbreds were used as the starting material for the "southern inbreds of maize" (SIM) population. Both populations showed a steady response to selection for silk maysin content, suggesting that additional gains for maysin could be attained (Widstrom and Snook 2001). Inbred lines were derived from cycle 5 (C5) of the SIM population and C6 of the EPM population by eight selfing generations.

The objective of this project was to determine which loci were responsible for the high maysin content in the EPM and SIM inbred lines. We identified candidate genes and determined whether novel loci were being detected in the QTL populations developed from the EPM and SIM inbreds. Candidate genes were tested for their role in maysin synthesis by expression analysis.

Materials and methods

Mapping populations

The EPM and SIM inbred lines were derived from C6 of the EPM population and C5 of the SIM population (Widstrom and Snook 2001) by eight self-pollinating generations. In each of these generations seed of a single ear was advanced ear-to-row and at least ten plants were self-pollinated. The selection of the ear for advancing was made primarily on ear size and seed number. The ten plants chosen for selfing were selected to maintain desirable morphological form. Although the plants were assayed for maysin content to confirm high maysin was maintained during inbred development, no selection for maysin content was performed during the self-pollination generations. One EPM inbred, one SIM inbred, and the inbred GT119 were used to develop the three F_2 populations (EPM × SIM, GT119 × EPM and GT119 × SIM). Visual observation and testcross analysis revealed that both the EPM and SIM inbred lines have a *p-wr* (functional, colorless pericarp and red cob) allele. The inbred line GT119 produces little to no maysin and has a *p-ww* (nonfunctional, colorless pericarp and colorless cob) allele (Szalma et al. 2005). Testcross and marker data confirmed that the *in1* allele from Coe G12# (*in1*) is present in the EPM inbred. A fourth QTL population was developed at the University of Missouri during the winter of 2003 by crossing one SIM cycle 8 self-pollinated (SIM8⊗) individual (selected for high maysin content) to an individual of the EPM inbred. A single F_1 plant was self-pollinated to derive the EPM × SIM8⊗ F_2 population.

Phenotypic analysis

The (EPM × SIM)F_2, (GT119 × EPM)F_2 and (GT119 × SIM)F_2 populations were grown during the summer of 2002 at the University of Missouri Genetics Research Farm (GRF) in Columbia, MO. Two-hundred eighty-eight individuals for each F_2 population were grown along with one row (20 individual plants) of each parental inbred and F_1 hybrid. Two-hundred forty individuals from the (EPM × SIM8⊗)F_2 along with parental and F_1 individuals were grown in the summer of 2003 at GRF. Primary ear shoots on inbred, F_1, and F_2 plants were covered prior to silk emergence to prevent pollination. Silks were collected from each F_2 plant at 2 days post-emergence from the husk. Individu-

als from the (EPM × SIM8⊗)F_2 population were self-pollinated after silk collection to derive $F_{2:3}$ families. Extract concentrations of maysin were determined by reversed-phase high-performance liquid chromatography (HPLC); (Snook et al. 1989, 1993) and expressed as percent fresh silk weight.

Genotypic analysis

Leaf tissue was harvested from parental, F_1 and F_2 individuals at the mid-whorl stage. The tissue was lyophilized, ground and DNA was extracted by the mixed cetyltrimethyl ammonium bromide procedure (Saghai-Maroof et. al. 1984). Simple sequence repeat (SSR) markers were used to gather genotypic information. Polymerase chain reaction (PCR) conditions were as follows: 40 ng DNA, 20 pmol of each primer, 5 µl Jumpstart Ready Mix REDTaq™ (Sigma–Aldrich, St. Louis, MO) and water for a total reaction volume of 10 µl. Cycling conditions were: ten cycles of (60 s at 95°C, 60 s at 65°C and 90 s at 72°C) with a 1°C decrement of annealing temperature per cycle until the annealing temperature was 55°C, followed by 25 cycles of (60 s at 95°C, 60 s at 55°C, 90 s at 72°C).

Statistical analysis

Deviation from normality of the phenotypic traits in the F_2 populations was tested with the Shapiro–Wilk statistic with PROC UNIVARIATE of SAS (Statistical Analysis System, Cary, NC) (SAS 1990). Linkage maps were generated by MAPMAKER/EXP, Version 3.0, Whitehead Institute, Cambridge, MA (Lander et al. 1987). A minimum likelihood of odds (LOD) score of 3.0 and a maximum distance of 50 cM were used to generate the final linkage maps. Composite interval mapping (CIM) was conducted with QTL Cartographer Version 1.16c (Basten et al. 1994, 2002). Cofactors for CIM were selected by forward/reverse regression at $P = 0.01$. For each trait-population combination, experimentwise significance thresholds of $P = 0.05$ were determined by analyzing 1,000 permutations of the data (Churchill and Doerge 1994). Analysis of all possible two-way interactions between loci was conducted using the SAS program EPISTACY (Holland 1998) with a significance threshold of $P = 0.001$.

Multiple locus models (MLM) were generated with SAS PROC GLM from markers detected by CIM and genetic interactions detected by EPISTACY. Individual markers nearest the peaks of the QTL and significant epistatic interactions were retained in the model if the $P < 0.05$ for the Type-III sum of squares. Genotype class means were obtained through the PROC MEANS procedure in SAS.

Direct mapping of candidate genes

Once QTL analysis was completed, candidate genes were determined for QTL. Gene specific primers for three candidate genes, *c2*, *whp1*, and *in1* (see electronic supplemental material, Table S1) were placed in the linkage maps of the mapping populations for which they reveled sequence tag site size polymorphisms. The location of the QTL relative to the candidate genes was determined by CIM.

Continued selection for high maysin

Twenty-six (EPM × SIM8⊗)F_2 individuals with the highest maysin levels were selected. An equal number of kernels from each selected $F_{2:3}$ ear was bulked and grown at the Illinois Crop Improvement Association site near Juana Diaz, Puerto Rico and random mated to form C1. A sample of 150 individuals from C1, 15 EPM individuals, 35 SIM8⊗ individuals, and 50 (EPM × SIM8⊗)F_2 individuals were grown at GRF during the summer of 2004. Silks were collected for maysin analysis and C1 plants were self-pollinated.

RNA collection and isolation

Three hundred and fifty (GT119 × EPM)F_2 individuals along with 20 EPM and 20 GT119 individuals were grown at GRF during the summer of 2003. At the mid-whorl stage, leaf punches were collected from F_2 individuals directly into 96-well PCR plates and DNA was extracted with the RED-Extract-N-Amp™ (Sigma–Aldrich, St. Louis, MO). Gene-specific primers for *c2*, *whp1*, *in1* were used to generate size polymorphisms to determine genotypes (Table S1). The SSR markers flanking the peak QTL for the *p* region (*bnlg1866* and *bnlg176*) were used to genotype the *p* locus. Plants with homozygous genotypes for *p*, *whp1* and *in1* were marked for silk collection. Primary ear shoots were covered before silk emergence and silks were collected between 12:00 and 14:00 on the same day they emerged from the shoot. The silks were immediately frozen in liquid nitrogen for transport to the laboratory, and stored at −80°C until extraction. After silk collection, F_2 ears were self-pollinated to generate $F_{2:3}$ families. Total RNA was isolated with Phase Lock Gel extractions (Eppendorf North America Inc., Westbury, NY). Genotypic bulks were made from the (GT119 × EPM)F_2 individuals homozygous for *p*, *in1* and *whp1*.

Phenotypic analysis for maysin concentration in (GT119 × EPM)F_2 genotypic bulks

$F_{2:3}$ families (20 plants) from each (GT119 × EPM)F_2 individual used in the genotypic bulks were grown in the summer of 2004 at GRF. Silks were collected from five $F_{2:3}$

plants at 2 days post-emergence and analyzed for maysin concentration as previously described, except that maysin concentrations were expressed as dry silk weight due to bulking $F_{2:3}$ individuals after lyophilization.

Real-time RT-PCR

All Taqman® probes and primer sets were designed with Primer Express software (ABI, Foster City, CA). As a control for total RNA, a primer probe combination for actin was utilized. Because the *whp1* and *c2* sequences are highly conserved, gene-specific primer and probe combinations for *whp1* and *c2* were designed from sequence of EPM and GT119 in the 3′ UTR of each gene (Fig. S1, Table S2). Primers and probes were diluted to 100 µM with DEPC-treated water and tested on DNA to ensure equal amplification. Real-time RT-PCR was conducted with the *Taq* Gold one-step RT-PCR master mix (ABI, Foster City, CA) with 30 µl total reaction volume. Standards were used at 200, 100, 50, 25, 12.5, 6.25 and 3.13 ng per reaction to derive a standard curve for normalization.

For real-time RT-PCR analysis, we used an ABI7000 Sequence Detection System (ABI, Foster City, CA). The PCR program consisted of 30 min at 50°C for reverse transcription, 10 min. at 94°C; then 40 cycles of 15 s. at 94°C for denaturing, and 1 min. at 60°C for annealing and elongation. The cycle thresholds were set in the log-growth phase at least four standard deviations above the baseline fluorescence values. Relative RNA amounts of *c2* or *whp1* transcript were determined by normalizing with actin transcript concentrations. The EPM inbred was used as a standard for reactions to determine *c2* and *whp1* specific message levels in the genotypic bulks.

Results

Phenotypic data

Maysin values for the EPM and SIM inbred were 3.7 and 1.5% fresh silk weight, respectively. GT119 averaged only 0.1% fresh silk weight maysin. Most inbred lines produce maysin levels in the range of 0.2–0.8% fresh silk weight (Snook et al. 1993; Lee et al. 1998). Not only are the maysin levels of the EPM and SIM inbreds higher than any of the starting materials for either population (Widstrom and Snook 2001), the EPM inbred maysin level is higher than any inbred tested to date. Although the distribution of maysin for F_2 individuals appears relatively normal in the (EPM × SIM)F_2 population (Fig. 1), it deviates from normality when tested by the Shapiro–Wilk statistic. For the (GT119 × EPM)F_2 and (GT119 × SIM)F_2 populations, the distribution is obviously skewed toward the lower values due to the large number of plants with very little maysin accumulation. In order to maintain the informative value of the individuals with more extreme values, we did not transform the data to correct for the deviations in distribution (Mutschler et al. 1996). The SIM8⊗ individuals synthesized large amounts of maysin (3.02% fresh silk weight).

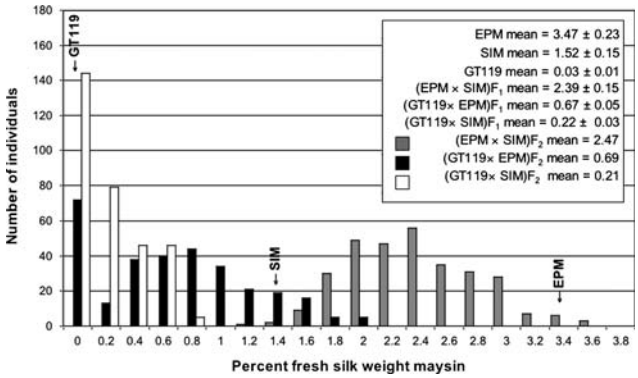

Fig. 1 Phenotypic distribution of maysin concentration in the (EPM × SIM)F_2, (GT119 × EPM)F_2, and (GT119 × SIM)F_2 populations

Genetic maps of QTL populations

Ten linkage groups were generated for the (EPM × SIM)F_2, (GT119 × EPM)F_2 and (GT119 × SIM)F_2 populations (Figs. S2, S3, S4), with total map distances of 1,664.9, 1,562.3 and 1,233.8 cM, respectively. Constructing a linkage map for (EPM × SIM8⊗)F_2 population proved to be difficult due to large, nonpolymorphic regions near the centromeres of chromosomes 6 and 9 (Fig. S5). Ninety-two polymorphic markers were anchored to the (EPM × SIM8⊗)F_2 population linkage map, in 12 linkage groups. SSR locations were consistent among all populations and with the IBM neighbors map (MaizeGDB, http://www.maizegdb.org).

QTL detection

In the (EPM × SIM)F_2 population, eight QTL for maysin were detected (Table 1). The largest effect QTL with the favorable allele (allele resulting in higher maysin levels) contributed by EPM was in bin 2.08 near marker *bnlg1316*. A candidate gene for this QTL is *whp1* which has been previously identified in maysin QTL studies (Byrne et al. 1998; Szalma et al. 2002, 2005). Four other QTL were detected with the positive allele contributed by the EPM parent; *bnlg1429* (bin 1.02), *umc1275* (bin 7.03) and *mmc0371* (bin 4.06). The QTL detected in bin 1.02 is consistent with the *p* locus (Byrne et al. 1996b, 1998; McMullen et al. 1998). A candidate gene for the QTL in bin 4.06 is *c2*, previously identified as a candidate gene for maysin or

Table 1 Quantitative trait loci for maysin from four maize populations

Position[a]			Contributing genetic effect[d]					Candidate gene
Chromosomes	cM/bin	Marker	LR[b]	Parent[c]	Additive	Dominant	R^2[e]	
(EPM × SIM)F_2 population								
1	20/1.02	*bnlg1429*	17.9	EPM	0.104	0.101	0.037	*P*
2	152/2.08	*bnlg1316*	41.2	EPM	0.197	−0.037	0.091	*whp1*
4	84/4.06	*mmc0371*	29.2	EPM	0.176	−0.022	0.074	*c2*[f]
5	89/5.03	*umc1705*	39.8	SIM	−0.206	−0.105	0.103	
5	154/5.07	*phi048*	21.3	EPM	0.133	0.019	0.042	
6	20/6.01	*bnlg1641*	21.1	SIM	−0.125	0.084	0.044	
7	91/7.03	*umc1275*	33.3	EPM	0.174	−0.054	0.076	*in1*
9	50/9.02	*bnlg1401*	40.8	SIM	−0.192	−0.044	0.090	*rem1*[f]
(GT119 × EPM)F_2 population								
1	66/1.02	*bnlg1866*	299.4	EPM	−0.592	0.035	0.610	*P*
2	38/2.03	*mmc0231*	25.0	EPM	−0.149	−0.019	0.038	
2	130/2.08	*bnlg1316*	44.7	EPM	−0.193	0.020	0.057	*whp1*
5	0/5.02	*bnlg1879*	17.6	EPM	−0.087	−0.122	0.019	
7	63/7.03	*umc1112*	62.6	EPM	−0.118	−0.043	0.024	*in1*
(GT119 × SIM)F_2 population								
1	51/1.02	*bnlg1866*	262.5	SIM	−0.234	0.063	0.639	*P*
4	8/4.00	*phi072*	15.1	GT119	0.033	0.043	0.028	
4	98/4.08	*umc1808*	27.2	GT119	0.070	−0.002	0.047	*c2*
7	28/7.01	*mmc0171*	25.5	SIM	−0.058	−0.060	0.053	
(EPM × SIM8⊗)F_2 population								
3	98/3.05	*mmc0022*	15.5	EPM	0.168	0.057	0.071	
4	116/4.06	mmc0371	18.1	EPM	0.169	0.014	0.060	*c2*[f]
8	64/8.02	*bnlg1352*	57.9	SIMC8	−0.294	0.012	0.228	
9	42/9.01	*umc1599*	17.9	SIMC8	−0.187	−0.119	0.108	*rem1*[f]

[a] The position in cM (relative to the terminal marker on the short arm) of the peak likelihood ratio, followed by bin location

[b] The likelihood ratio at the QTL peak

[c] The allele that increases the trait

[d] Additive and dominance effects are reported relative to EPM in the (EPM × SIM)F_2 population, GT119 in the (GT119 × EPM)F_2 population, and GT119 in the (GT119 × SIM)F_2 population

[e] R^2 is the amount of phenotypic variance for the trait explained by that locus

[f] The QTL detected in both the (EPM × SIM)F_2 and (EPM × SIM8⊗)F_2 populations

CEW larvae antibiosis in four maize populations (Byrne et al. 1998; McMullen et al. 2001; Szalma et al. 2002). A candidate gene for the QTL near bin 7.03 is *in1* which was initially implicated in maysin accumulation in the (W23a1 × GT119)F_2 population (McMullen et al. 2001) and subsequently detected in two additional populations (Bushman et al. 2002; Szalma et al. 2002). Three QTL were detected in the (EPM × SIM)F_2 population with the favorable allele contributed by the SIM parent. These include the QTL with the largest effect in the (EPM × SIM)F_2 population in bin 5.03 near marker *umc1705* ($R^2 = 0.10$) and a region in bin 9.02 near marker *bnlg1401*. The location of the 9.02 QTL is consistent with the reported location of *rem1* (Byrne et al. 1996b, 1998).

In the (GT119 × EPM)F_2 population, five main effect QTL for maysin were detected (Table 1). The favorable allele at all five QTL came from EPM, the high maysin parent. The majority of phenotypic variance ($R^2 = 0.61$) in this population was explained by the QTL near marker *bnlg1866* (bin 1.03) which is consistent with the location of *p*. Two of the remaining QTL detected were located in bins 2.08 and 7.03. Candidate genes for these QTL are *whp1* (bin 2.08) and *in1* (bin 7.03). Both *in1* and *whp1* were candidate genes for maysin content in the (EPM × SIM)F_2 population. The additional QTL (bins 2.03 and 5.02) explain a small amount of phenotypic variance.

In the (GT119 × SIM)F_2 population, four main effect QTL were detected (Table 1). For two of the QTL, the

favorable allele was contributed by SIM. The QTL that contributed the majority of phenotypic variance (R^2 = 0.639) was in bin 1.02 near the marker *bnlg1866* and consistent with the position of the *p* region. The largest QTL (R^2 = 0.047) with the positive allele contributed by GT119 is located in bin 4.08 near marker *umc1808*. This location is consistent with *c2*, also a candidate gene in the (EPM × SIM)F_2 population.

A fourth QTL population, the (EPM × SIM8⊗)F_2 population was analyzed to determine what genes were responsible for the large amount of maysin in the SIM8⊗ individual (3.0% fresh silk weight) as compared to the SIM C5 inbred (1.5% fresh silk weight). The QTL detected in the (EPM × SIM8⊗)F_2 population are listed in Table 1 and include a marker near the *c2* locus (*mmc0371)* and the *rem1* locus (*umc1599*) which were also detected in the (EPM × SIM)F_2 population.

Multiple locus models

Epistatic interactions have been shown to have significant effects on maysin concentration in maize silks (McMullen et al. 2001; Szalma et al. 2002; Bushman et al. 2002). Significant epistatic interactions for maysin were detected in all three populations (Table 2). In the (EPM × SIM)F_2 population, the MLM for maysin explained 62.4% of the total phenotypic variance. All of the main effect QTL detected by CIM remained in the model with the exception of two loci, *bnlg1429* (bin 1.02) and *phi048* (bin 5.07). Three interactions were included in the model (Table 2).

The MLM for the (GT119 × EPM)F_2 population for maysin synthesis accounted for 76.4% of the total phenotypic variance. All main effect QTL detected by CIM remained significant in the model and the model included one interaction involving the main effect QTL near marker *bnlg1866* and another locus, *umc1580* (bin 2.04).

In the (GT119 × SIM)F_2 population, the MLM for maysin explained 64.7% of the total phenotypic variance. All of the main effect QTL detected by CIM remained in the model with the exception of *mmc0171* (bin 7.01). Two interactions involving the *p* locus, were included in this model. One interaction is *bnlg1520* × *umc1568* (bin 2.09 × 1.02). As previously mentioned, *umc1568* is near *p* and the location of *bnlg1560* is consistent with the location of *whp1*. The second interaction is *bnlg439* × *umc1543* (bin 1.03 × 7.04).

Maysin concentrations for the genotypic bulks

Although the positions of QTL are consistent with candidate loci discussed above the significance intervals are large and contains numerous genes, therefore more experimental evidence is necessary to support the role of these candidates in controlling maysin levels. The role of the *p* locus has been previously confirmed by transformation studies (Grotewold et al. 1994, 1998; Zhang et al. 2003; Cocciolone et al. 2005). Previous studies for *c2* and *whp1* utilized known mutants at these loci to support candidate gene identification (Szalma et al. 2002). Therefore, the candidate genes *c1*, *whp1* and *in1* were tested by expression analysis and direct mapping onto the QTL populations.

Table 2 Multiple locus models for maysin accumulation in three maize populations

Marker	Bin	*P*	Candidate gene
(EPM × SIM)F_2 population			
bnlg1316	2.08	0.0001	*whp1*
mmc0371	4.06	0.0017	*c2*
umc1705	5.03	0.0001	
bnlg1641	6.01	0.0035	
umc1275	7.03	0.0001	*in1*
bnlg1401	9.02	0.0001	*rem1*
bnlg1063[a]	3.04	0.0410	
umc1766[a]	5.01	0.6576	
bnlg1447[a]	4.08	0.1896	
umc1567[a]	7.03	0.8068	
phi328175[a]	7.04	0.3103	
umc2208[a]	6.00	0.2814	
bnlg1063 × *umc1766*	3.06 × 5.01	0.0001	
bnlg1447 × *umc1567*	4.08 × 7.03	0.0329	
phi328175 × *umc2208*	7.04 × 6.00	0.0056	
R^2 = 0.624			
(GT119 × EPM)F_2 population			
bnlg1866	1.02	0.0001	*P*
mmc0231	2.03	0.0001	
bnlg1316	2.08	0.0001	*whp1*
bnlg1879	5.02	0.0025	
umc1112	7.03	0.0036	*in1*
bnlg1580[a]	2.04	0.0026	*qtl2*
bnlg1866 × *bnlg1580*	1.02 × 2.04	0.0005	*P* × *qtl2*
R^2 = 0.764			
(GT119 × SIM)F_2 population			
bnlg1866	1.02	0.0001	*P*
phi072	4.00	0.0038	
umc1808	4.08	0.0001	*c2*
bnlg439	1.03	0.9451	
umc1543	7.04	0.6697	
bnlg1520	2.09	0.3785	*whp1*
umc1568	1.02	0.0113	
bnlg1520 × *umc1568*	2.09 × 1.02	0.0058	*whp1* × *P*
bnlg439 × *umc1543*	1.03 × 7.04	0.0001	*P* × *?*
R^2 = 0.647			

[a] Denotes markers added to MLM only because they were involved in an epistatic interaction

The (GT119 × EPM)F_2 population was chosen for further analysis of the effect of *in1* on *whp1* transcription because *c2* was not detected as a QTL in this population (Table 1). Silks were collected from (GT119 × EPM)$F_{2:3}$ individuals and bulked for HPLC analysis (Fig. 2a). Whenever the genotypic bulk contains the nonfunctional (GT119) *p* allele, there are very low maysin levels (less than 0.05% dry silk weight). When the EPM *p* allele is present, the maysin levels increase more than ten fold. The BAA (EPM *p* allele, GT119 *in1* and *whp1* alleles) bulk has a maysin average of 4.98 ± 2.9% dry silk weight maysin. Adding either the EPM allele of *in1* or *whp1* (BBA or BAB) increases the maysin values (7.88 ± 2.2 and 8.55 ± 2.7% dry silk weight, respectively). However, there is no statistically significant difference between the BAB, BBA and BBB genotypic groups. Therefore, having both the EPM copy of *whp1* and *in1* (8.79 ± 2.9% dry silk weight) is not statistically different for maysin content than having either the EPM *whp1* or *in1* allele alone.

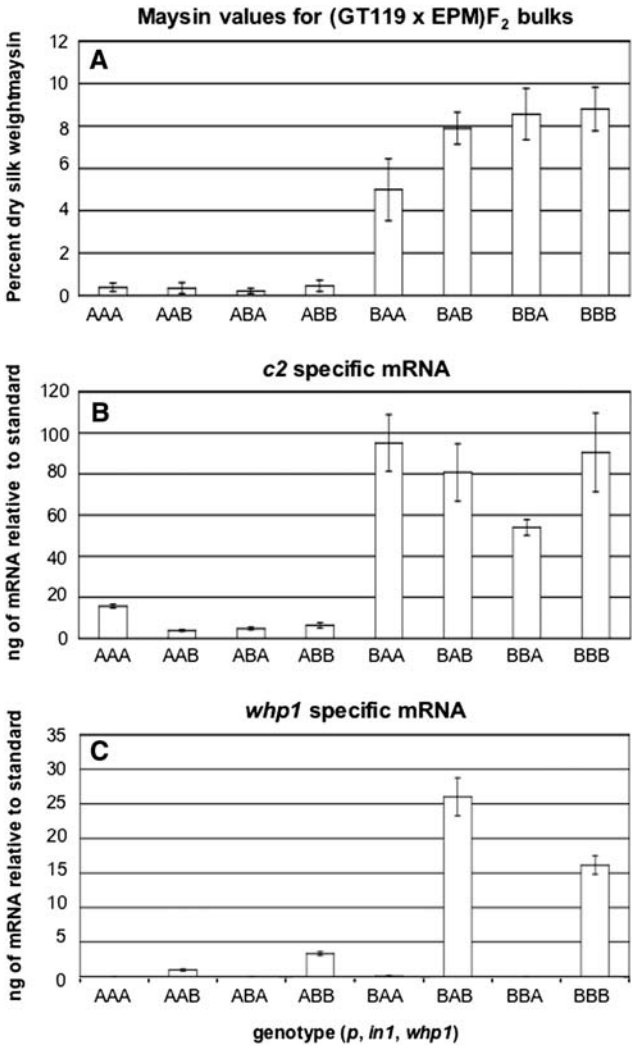

Fig. 2 Maysin and mRNA levels of (GT119 × EPM)F_2 silk bulks based on the genotypes at *p*, *in1* and *whp1*. The letters A and B designate GT119 and EPM alleles, respectively. **a** Silk maysin levels in percent dry silk weight for genetic bulks. **b** The relative expression of *c2* among the genotypic bulks. **c** The relative expression of *whp*1 among the genotypic bulks

c2 specific mRNA

The *c2* specific mRNA quantities for the genotypic bulks from the (GT119 × EPM)F_2 were determined (Fig. 2b). When the genotypic bulk contains the nonfunctional *p* allele, there is a low detectable level of *c2* expression. However, when the EPM allele of *p* is present, the amount of *c2* specific mRNA is significantly higher. The positive effect of the EPM *p* allele on *c2* mRNA supports the previous literature that *p* regulates *c2* transcription (Bruce et al. 2000) and the expression data support both *p* and *c2* as the genes underlying the QTL from chromosomes 1 and 4.

whp1 specific mRNA

The (GT119 × EPM)F_2 genotypic bulks were also tested for *whp1* specific mRNA levels (Fig. 2c). When the bulks contain the GT119 *whp1* allele, there is little or no *whp1* message compared to the bulks that contain the EPM *whp1* allele. This result supports *whp1* itself as a candidate gene due to some structural feature in the GT119 *whp1* allele (cis effect).

When we compared the bulk with the GT119 *p*, GT119 *in1*, and EPM *whp1* alleles (bulk AAB) to the bulk with the GT119 *p* allele, EPM *in1*, and EPM *whp1* alleles (bulk ABB) the bulk with the EPM allele of *in1* has a higher level of *whp1* transcript level. However when comparing the bulk containing the EPM *p*, GT119 *in1*, and EPM *whp1* alleles (bulk BAB) to the bulk containing the EPM *p*, EPM *in1*, and EPM *whp1* alleles (bulk BBB), the bulk with the EPM *in1* allele has a lower *whp1* transcript level than the bulk with the GT119 *in1* allele. Therefore the role of *in1* on *whp1* transcript level in silk tissue is unclear.

Comparison of bulks containing the EPM *p* and EPM *whp1* alleles to bulks containing the GT119 *p* and EPM *whp1* alleles reveals that there is a significantly higher *whp1* mRNA level when the bulks contain the EPM *p* allele. This significant difference of *whp1* expression between the EPM and GT119 *p* alleles indicates that *p* is important for *whp1* transcription in maize silk tissue.

Direct mapping of candidate loci

Gene specific primers for *in1* were used to test if the *in1* locus is located within the QTL on chromosome 7 in the (EPM × SIM)F_2 and (GT119 × EPM)F_2 populations (Fig. S6). Not only was the *in1* locus not significant for silk maysin content, but it was two markers and 43 cM

away from the QTL in the (EPM × SIM)F_2 population and four markers and 45 cM way from the QTL in the (GT119 × EPM)F_2 population. The *c2* locus was mapped in the (GT119 × SIM)F_2 population next to the marker designated as nearest to the QTL by CIM (Fig. S7). Also the map position of the *whp1* locus was consistent with the QTL on chromosome 2 in the (GT119 × EPM)F_2 population (Fig. S7).

Discussion

Novel QTL

Were novel QTL detected from the very high maysin lines? All of the QTL detected for maysin content in the (EPM × SIM)F_2, (GT119 × EPM)F_2, and (GT119 × SIM)F_2 populations were within the confidence intervals of QTL detected in previous QTL experiments (Byrne et al. 1996a, b; Lee et al. 1998; McMullen et al. 1998, 2001; Szalma et al. 2002; Bushman et al. 2002; Cortés-Cruz et al. 2003) with the exception of a small effect (R^2 = 1.9%) QTL detected in bin 5.02 in the (GT119 × EPM)F_2 population. Because only 11 total QTL were detected in the (EPM × SIM)F_2, (GT119 × EPM)F_2 and (GT119 × SIM)F_2 populations, it may be possible that other novel QTL were not detected; however, in the MLM for all populations, at least 60% of the phenotypic variance was explained. Therefore, our data support the conclusion that very high maysin levels in the EPM and SIM materials are by combination of QTL known from analysis of standard maize inbred lines.

The *p* locus

It is not surprising that the *p* locus is detected as a QTL in this study. The protein encoded by the *p* locus is a *myb* transcription factor and regulates structural genes within the phenylpropanoid and flavonoid pathways (Bruce et al. 2000). The regulation of maysin synthesis by the *p* locus was demonstrated by transforming BMS (Black Mexican Sweet which contains the *p1*-www allele) callus cells with a functional *p1* allele (Grotewold et al. 1994, 1998). The introduction of a functional *p* allele resulted in increased synthesis of C-glycosyl flavones related to maysin and 3-deoxy flavonoid precursors of phlobaphenes. More recently, Zhang et al. (2003) and Cocciolone et al. (2005) determined that introducing specific *p1* alleles in transgenic plants directly affects maysin levels.

Chalcone synthase loci

The *whp1* region on the long arm of chromosome 2 and the *c2* region on the long arm of chromosome 4 have been identified as candidate genes for maysin accumulation (Byrne et al. 1998; Lee et al. 1998; Szalma et al. 2002). Increasing CHS activity would increase the amount of chalcone and therefore potentially all of the products of the flavonoid pathway branches, including maysin.

The QTL near *mmc0371* (bin 4.06) in the (EPM × SIM)F_2 population and the QTL near marker *umc1808* (bin 4.08) in the (GT119 × SIM)F_2 population are consistent with the location of *c2*. This locus accounts for 7.5% of the variance in the (EPM × SIM)F_2 population and 4.7% in the (GT119 × SIM)F_2 population. The positive allele for *c2* is contributed by EPM in the (EPM × SIM)F_2 population and by GT119 in the (GT119 × SIM)F_2 population. The lack of detection of *c2* as a QTL in the (GT119 × EPM)F_2 population indicates that the *c2* alleles from EPM and GT119 are statistically equivalent. One interaction detected in the (EPM × SIM)F_2 population involved one locus consistent with *c2*, near marker *umc1447* (bin 4.08) and the other locus near marker *umc1567* (bin 7.03). The real-time RT-PCR data indicate that the *p* locus affects *c2* transcript levels (Fig. 2b), supporting previous reports that *c2* mRNA is regulated by *p* (Bruce et al. 2000; Bushman et al. 2002).

The QTL near *bnlg1316* (bin 2.08) in the (EPM × SIM)F_2 and the (GT119 × EPM)F_2 population is consistent with the location of *whp1*. This locus accounts for 5.7% of the variance in the (GT119 × EPM)F_2 population and 9.1% in the (EPM × SIM)F_2 population. The positive allele at the *whp1* locus is contributed by EPM in the (EPM × SIM)F_2 and the (GT119 × EPM)F_2 populations. The *whp1* locus is supported as a candidate gene by the real-time RT-PCR data (Fig. 2c). The data suggest that GT119 may have a significantly less functional *whp1* allele than EPM. Through association analysis, Szalma et al. (2005) detected a sequence divergence in *whp1* which had a significant association with maysin content in maize silks. The sequence divergence was in the 5′ UTR of *whp1* and Szalma et al. (2005) hypothesized the sequence divergence may affect transcription. Although this would be a logical explanation for the lack of transcription of the GT119 *whp1* allele, the sequence reported for GT119 in the association analysis causes more maysin. The specific cause for the lack of transcription of the GT119 *whp1* allele in this study is unknown.

Although *whp1* was not detected as a main effect QTL in the (GT119 × SIM)F_2 population, a marker with location consistent with *whp1* was detected in an interaction with a marker near *p*. An interaction between *p* and *whp1* has not been previously reported for maysin synthesis, but is supported by the real-time RT-PCR results where a significant difference in *whp1* transcript levels was dependent on both the *whp1* allele and the functional *p* allele from EPM.

The combination of expression data and mapping data strongly support *c2* and *whp1* as candidate loci for QTL for

maysin synthesis. This result is consistent with the prior results using mutants at these loci and association analysis for *whp1* for the involvement of these loci in maysin synthesis.

The *in1* locus

The *in1* locus has been shown to promote expression of *whp1* when *in1* is in the homozygous recessive state (Coe 1957; Coe et al. 1998; Burr et al. 1996; Franken et al. 1991). Although the RNA data does not support *in1* as a candidate gene in the (EPM × SIM)F_2 or (GT119 × EPM)F_2 populations, it is important to note that the first observation of the effect of *in1* on *whp1* expression was in aleurone tissue, where *p* is not expressed. The direct mapping data for the *in1* locus also does not support *in1* as the candidate gene for the QTL on chromosome 7. With these results we eliminate *in1* as the candidate for the QTL on chromosome 7 and do not currently have a candidate gene for this QTL.

Genetic basis of high maysin

The *p* locus, *whp1*, and *c2* are candidate genes for QTL detected for maysin accumulation. While there are always questions of assigning candidates based on QTL position, our RT-PCR results, interpreted in the framework of existing knowledge on the regulation of flavone synthesis, provide convincing support that these genes underlay the QTL. Variation at all of these loci can be viewed as affecting CHS. The detection of these loci suggests high maysin levels are a response to increase flux through the flavone pathway by an increase in CHS activity. Additional QTL may function within the flavone pathway or within other branches of the flavonoid pathway. These loci may have a smaller effect than *p*, *whp1*, and *c2* because they may affect loci downstream of the CHS function, or in other pathways sharing intermediates with flavonoid synthesis. While we initially thought that *in1* was a candidate gene for maysin synthesis through it reported regulatory role on *whp1*, real-time RT-PCR experiments did not support this hypothesis. Instead, our data support the idea that *whp*1 is transcriptionally regulated in maize silks by *p*, not *in1*, in silk tissue.

Loci with small genetic effects may be more easily detected in the (EPM × SIM)F_2 population due to similar *p* regions. One of the most interesting findings is that positive alleles for all the QTL were not contributed by the same parent, especially in the (EPM × SIM)F_2 population. The data suggest that the EPM and SIM populations achieved their high maysin levels by accumulating different alleles at both structural and regulatory QTL. The accumulation of alleles at different loci also suggests that even higher maysin levels can be attained. Further evidence of higher maysin potentials became apparent upon analysis of a fourth

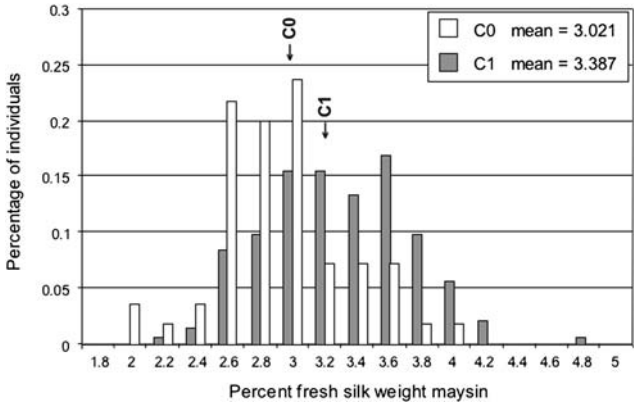

Fig. 3 Distribution of maysin concentrations for individuals of C0 and C1 of selection in the (EPM × SIM8⊗) population. C0 is the (EPM × SIM8⊗)F_2 population and C1 is the random mated progeny of 26 individuals selected from C0. The *y*-axis represents the percentage of individuals in the population

QTL population, the (EPM × SIM8⊗)F_2 population. This population was subjected to one cycle of selection to determine what maysin concentrations could be attained by combining the alleles from the EPM and the SIM8⊗ individual. The population distributions are shown in Fig. 3. The population mean for C0 (the sample of the (EPM × SIM8⊗)F_2 population) was 3.02% fresh silk weight maysin. The population mean for C1 was 3.39% fresh silk weight maysin. These results indicate a 0.37% increase in the population mean in one cycle, which is higher than the response previously reported for either the EPM (0.22% ± 0.014 per cycle) or SIM (0.19% ± 0.012 per cycle) populations individually (Widstrom and Snook 2001). Therefore, even higher maysin levels are possible when making selections within the new (EPM × SIM8⊗)F_2 population.

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