

Haplotype frequencies at the *miR156* and *miR172* transcript regions show a significant response to divergent recurrent selection for vegetative phase change in maize

Jessica Rutkoski, Eric Riedeman, William Tracy, and Shawn Kaeppler.

Abstract

Sweet corn (*Zea mays* L.) plants that transition from juvenility to the adult phase earlier during development are generally more resistant to common rust (*Puccinia sorghi*), a disease that causes substantial yield loss in sweet corn. This developmental transition is called vegetative phase change (VPC). VPC is regulated in part by *miR156* and *miR172*. We found that natural variation at the *miR156* and *miR172* transcript regions are important in determining variation in the timing of VPC. We sequenced the *miR156* and *miR172* transcript regions in populations originating from the same source population and divergently selected for eight generations for early and late VPC. For both the *miR156* and *miR172* transcript regions we detected four distinct haplotypes. Overall and individual haplotype frequencies showed significant responses to selection for both early and late VPC. These data contribute to the identification of important loci involved in VPC. Understanding the genetic basis for VPC will facilitate maize breeding aimed at combining early VPC with other desirable traits.

Jessica Rutkoski Genetics
Author Name/Major

Jessica Rutkoski
Author Signature

5/10/09
Date

Bill Tracy, Agronomy
Mentor Name/Department

Bill Tracy
Mentor Signature

COVER SHEET

TITLE: Haplotype frequencies at the *miR156* and *miR172* transcript regions show a significant response to divergent recurrent selection for vegetative phase change in maize

AUTHOR'S NAME: Jessica Rutkoski

MAJOR: Genetics

DEPARTMENT: Genetics

MENTOR: Bill Tracy

DEPARTMENT: Agronomy

MENTOR(2): Shawn Kaeppler

DEPARTMENT(2):Agronomy

YEAR: 2009

(The following statement must be included if you want your paper included in the library's electronic repository.)

The author hereby grants to University of Wisconsin-Madison the permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part in any medium now known or hereafter created.

Haplotype frequencies at the *miR156* and *miR172* transcript regions show a significant response to divergent recurrent selection for vegetative phase change in maize

Jessica Rutkoski, Eric Riedeman, William Tracy, and Shawn Kaeppler.

Abstract

Sweet corn (*Zea mays* L.) plants that transition from juvenility to the adult phase earlier during development are generally more resistant to common rust (*Puccinia sorghi*), a disease that causes substantial yield loss in sweet corn. This developmental transition is called vegetative phase change (VPC). VPC is regulated in part by *miR156* and *miR172*. We found that natural variation at the *miR156* and *miR172* transcript regions are important in determining variation in the timing of VPC. We sequenced the *miR156* and *miR172* transcript regions in populations originating from the same source population and divergently selected for eight generations for early and late VPC. For both the *miR156* and *miR172* transcript regions we detected four distinct haplotypes. Overall and individual haplotype frequencies showed significant responses to selection for both early and late VPC. These data contribute to the identification of important loci involved in VPC. Understanding the genetic basis for VPC will facilitate maize breeding aimed at combining early VPC with other desirable traits.

Introduction:

Common rust is the most prevalent disease afflicting sweet corn (*Zea mays* L.) grown in the upper Midwest. Rust is caused by the fungal pathogen *Puccinia sorghi*, which is often controlled by applying fungicides to crops and by growing sweet corn with the *Rp1-D* allele conferring genetic resistance. Since 1999, new races of *Puccinia sorghi* have appeared in the Midwest that are able to infect sweet corn plants with the *Rp1-D* allele making this resistance gene ineffective (Pataky et al. 2001). This study investigates a new form of genetic resistance

that exploits partial adult plant resistance to rust that occurs in adult sweet corn plants. The two vegetative phases in maize, juvenile and adult, are distinct in that adult leaves have a thicker cuticle and juvenile leaves have more epicuticular wax (Poethig, 1990). Juvenile leaves are more susceptible to *P. sorghi*. Plants with early or late vegetative phase change can be selected to decrease or increase the duration of the juvenile phase; those with a longer juvenile phase experience greater susceptibility to common rust and insects (Basso et al., 2008; Abedon and Tracy, 1996).

Vegetative phase change (VPC) in maize is regulated in part by *microRNA156* (*miR156*) and *microRNA172* (*miR172*). MicroRNAs are small RNA sequences that cleave complementary

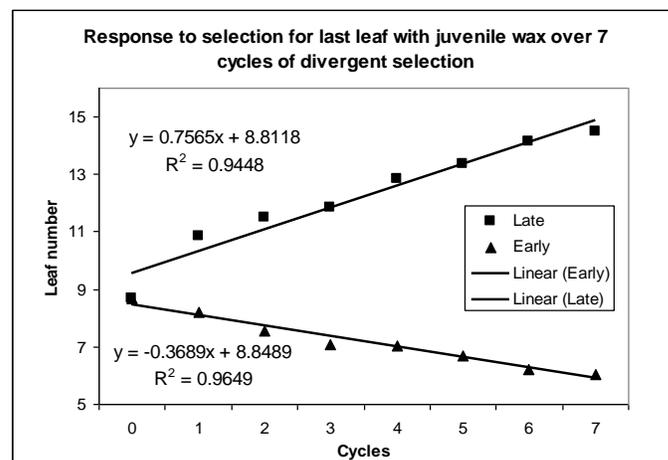


Figure 1: The timing of VPC has shown a linear response over seven cycles of divergent recurrent selection from an original population of non-*Cg1* maize. The last leaf with juvenile wax measures the timing of VPC.

mRNA sequences, therefore reducing the level of the protein product the target mRNA codes for.

Overexpression of *miR156* increases the duration of the juvenile phase (Chuck et al., 2007).

miR156 cleaves target mRNA that code for specific transcription factors that promote

transcription of developmental genes. The transcription factors are therefore less numerous and

less of the corresponding developmental gene is expressed. (Rhodes et al. 2002). *gl15* is an

essential gene for juvenile leaf trait expression. *miR172* down regulates the juvenility gene *gl15*

by cleaving the transcript of *gl15*. This promotes the transition to the adult vegetative phase (Lauter et.al, 2005).

From an original Minn 11 sugary1 sweet corn population, Professor Bill Tracy has used divergent recurrent selection selecting VPC in the late and early direction for eight cycles in an ongoing study. The timing of VPC has been very responsive. (Figure 1) (Riedeman et al., 2008). Morphological changes have resulted and susceptibility to rust has increased in the cycles selected in the late phase change direction. In this experiment, we used sequencing to test the hypothesis that the haplotype frequencies at the *miR156* and *miR172* transcript regions were altered in response to selection and whether the natural variation at the *miR156* transcript region and the *miR172* transcript region is important in determining the observed variation of the timing of vegetative phase change (VPC). Understanding the genetic basis for regulation of VPC in maize will allow breeders to produce maize that combines high quality traits with early VPC, conferring partial yet durable resistance to rust (Basso et al., 2008, Abedon and Tracy, 1996).

Materials and Methods

Population Development: Minn 11, a sugary1 sweet corn population, formed cycle 0 (C0) of a divergent recurrent selection program. While the parents of Minn 11 included both Corn Belt Dent and sweet corn germplasm, the exact parents are unknown. Approximately 150 C0 plants were self-pollinated, harvested, and planted in family rows. Family rows were evaluated for the average last leaf with juvenile wax. The earliest and latest 20% for last leaf with juvenile wax intermated using bulked pollen from the selections. A minimum of 100 ears was obtained for each direction of selection. Equal amounts of seed from these ears were pooled to form balance bulks for each direction. Remnant seed was planted ear to row for the next cycle of selection. This process was repeated through eight cycles of selection in both directions. The original

population (C0), cycle 8 in the early direction of selection (C8E), and cycle 8 in the late direction of selection (C8L) were the populations evaluated for haplotype frequency in this experiment.

Leaf harvest and preparation: We grew 120 plants from C0, and the C8E and C8L. Immature leaves were harvested, freeze-dried and pulverized for DNA extraction.

DNA extraction: We isolated DNA using the DNA isolation using CTAB protocol (Kidwell, K.K., and T.C. Osborn, 1993). We measured DNA concentration using a nano-drop spectrophotometer.

Population parentage testing: Four potential parents of the C0 population, B73, Pa405, Ia453, and II125b, were screened using microsatellite (SSR) markers. DNA from the four potential parents and 96 individuals from C0 were PCR amplified with random SSR marker primers.

Polymorphisms were screened using the Mega-Gel protocol for polyacrylamide gel electrophoresis. Each gel mix contained 75 ml 1x TBE, 22.5 ml 40% acrylamide stock 1.05 ml 10% APS, 120 μ l TEMED, and 51 ml of ddH₂O. PCR amplified samples were stained using Type IV loading dye (6x) and 1.25 μ l of a 1/100 dilution of Gel Red per 10 μ l. Stained samples were run on a gel at 7 watts for 2 hours, a second set of samples was run then loaded and run for 2 hours. Banding patterns on the gel were visualized and photographed using a UV light box.

DNA amplification: Three bulks of DNA, 100ng from each individual within its respective population, were made one each for C0, C8L, and C8E. We used and optimized PCR protocol to amplify each DNA bulk. We used a GoTaq[®] Hot Start Polymerase kit from Promega. Our reaction mixes contained 2 μ l of 10x colorless buffer, 0.4 μ l dNTP, 0.65 μ l forward and reverse primers, 0.1 μ l HotStar Taq, 0.8ng dry DNA and 16.05 μ l ddH₂O, total reaction amount was 20 μ l. We used the *miR156* forward primer GTGGCGTCAACAACATTACG , and reverse primer AAGGCATACCCCAACAATCA which flanks a region about 1900 bps downstream from the

miR156 transcript region. We used the *miR172* forward primer ACAAAGGCCAAGCGCTACTA, and reverse primer CACATGGGTGACGATGCTAC which flanks a region about 1500 bps downstream of the *miR172* transcript region. We amplified each bulk once using primers for the *miR156* transcript region and again for the *miR172* transcript region (six PCR products total). Specific priming and amplification of these regions was confirmed using gel electrophoresis on 1% agarose gels. Resolution of more than one band on the gel indicated non-specific priming or DNA degradation.

DNA purification: We cut the primer specific bands out of the 1% agarose gel to ensure isolation of primer specific DNA. Used a QIAquick spin column kit to remove primers, nucleotides, enzymes, mineral salts, agarose, ethidium bromide, and other impurities from the DNA samples. Purified DNA was concentrated with EtOH DNA precipitation, and resuspended in 10ul TE.

Cloning into bacteria: We ligated each of the six purified PCR products into a pGEM-T Easy Vector using T4 DNA ligase. Ligation of control insert DNA into the vector was used as a positive control and ligation of the empty vector without insert DNA was used as a negative control. We prepared 2 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction and equilibrated the plates to room temperature prior to plating. We transformed JM109 High Efficiency Competent Cells by mixing 2ul of each ligation reaction with 50ul of competent cells. The mix was placed on ice for 20 minutes, heat-shocked for 45-50 seconds in a 42°C water bath, and returned to ice for 2 minutes. We incubated the transformed cells for 1.5 hours at 37°C with shaking and plated 100ul of each culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. Plates were incubated overnight at 37°C. White colonies contained inserts. Individual white colonies

were transferred to a replicate plate and a well in a 96 well PCR plate. One plate was designated for each of the 6 transformation reactions.

Clean up and Sequencing: We PCR amplified DNA from transformed colonies. PCR products were diluted 2:40 with TE. We prepared our each of the six 96 well plates for dye-terminator sequencing using the BigDye terminator kit containing fluorescently labeled dideoxy terminators. Only the plate's corresponding forward primers were used. The reactions were run on a thermocycler using the BigDye protocol. DNA was cleaned up and sequenced at the UW Madison Biotechnology DNA Sequence Laboratory.

Analysis: DNA sequences were aligned using BioEdit software and significance in changes of haplotype frequency was determined using SAS. We ran two-way chi-square tests when over 80% of expected values were over 5. Fishers exact tests used when this criteria was not met. The significance level for both tests was set at $\alpha = 0.05$.

Results:

Population parentage:

We aimed to identify the parents of C0 in order to develop SNP markers that could be used to screen C0, C8E, and C8L for haplotype frequency changes. We found that SSR Markers Bnlg-1144 and Umc-1014 produced bands present in C0 that were absent in the potential parents. We thus eliminated B73, Pa405, Ia453, and Il125b as the parents of C0 (Figure 2). We therefore proceeded to detect haplotype frequency changes between C0, C8E, and C8L via sequencing.

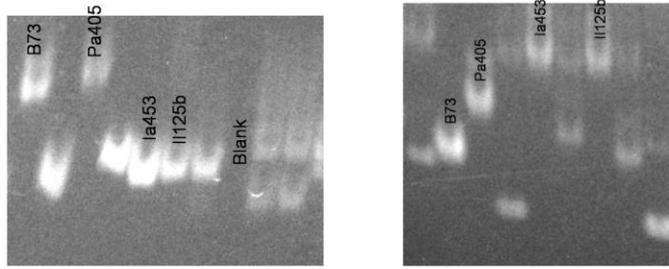


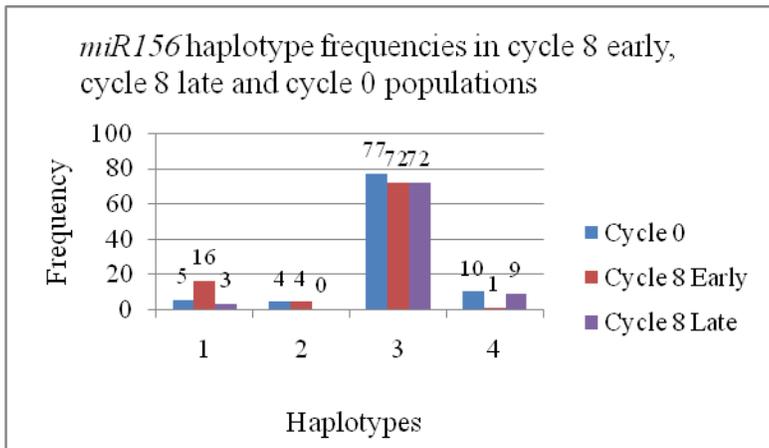
Figure 2: SSR markers umc-1014 (left) and bnlg-1144 (right) resolve bands in the C0 individuals that are not present in the potential parents, B73, Pa405, Ia453, and II125b. These candidates are thus eliminated as the parents of C0.

From sequence data at the *miR156* transcript region six single nucleotide polymorphisms (SNPs) were found. Five of the SNPs were organized into four distinct haplotypes. Overall haplotype frequencies varied significantly between C0 and C8E as well as between the C8L and C8E populations (Table 1). Using a two-way chi-square we found that haplotype frequencies between C8E and C8L were significantly different ($p=0.0003$). Using a Fisher's exact test we found that haplotype frequencies were significantly different between the C0 and C8E ($p=0.0026$), but were not significantly different between C0 and C8L ($p=0.3028$). These data suggest that the *miR156* transcript region is a target for selection for early VPC.

From sequence data at the *miR172* transcript region eight SNPs and nine insertion/deletion polymorphisms were found. All these polymorphisms were organized into four distinct haplotypes. Overall haplotype frequencies varied significantly between C0 and C8L as well as between the C8L and C8E populations (Table 1). Using a two-way chi-square test, we found that haplotype frequencies were significantly different between C8E and the C8L ($p=0.0002$), but were not significantly different between C0 and the C8E ($p=0.1013$). Using a Fisher's exact test, we found that haplotype frequencies were significantly different between C0 and the C8L ($p=0.0231$). These data suggest that the *miR172* transcript region is a target for selection for late VPC.

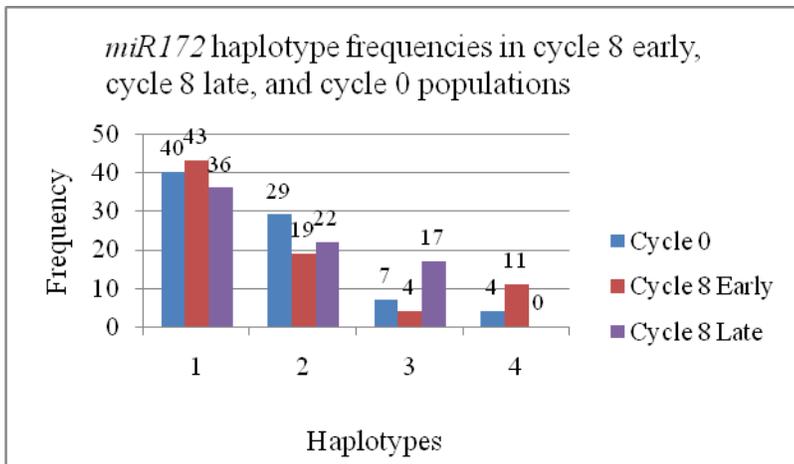
Comparison pairs	<i>miR156</i> transcript region	<i>miR172</i> transcript region
Cycle 8 early vs. Cycle 0	p=0.0026	p=0.1013
Cycle 8 late vs. Cycle 0	p=0.3028	p=0.0231
Cycle 8 late vs. Cycle 8 early	p=0.0003	p=0.0002

Table 1: Statistical significance of overall haplotype frequency among all possible comparisons of C0, C8E and C8L



	C0	C8E	C8L
haplotype 1	5	16	3
haplotype 2	4	4	0
haplotype 3	77	72	72
haplotype 4	10	1	9

Figure 3: Haplotype frequencies at the *miR56* transcript region for C80, C8E, and C8L



	C0	C8E	C8L
haplotype 1	40	43	36
haplotype 2	29	19	22
haplotype 3	7	4	17
haplotype 4	4	11	0

Figure 4: Haplotype frequencies at the *miR172* transcript region for C80, C8E, and C8L

Frequencies of individual haplotypes at the *miR156* and *miR172* transcript regions varied between C0, C8E, and C8L (Figure 3, Figure 4). Using two-way chi-square tests, we found that the frequency of haplotype one at the *miR156* transcript region was significantly increased by selection for early VPC ($p=0.0105$) and was significantly different between C8E and C8L. The frequency of haplotype four at the *miR156* transcript region was significantly decreased by selection for early VPC ($p=0.096$) and was significantly different between C8E and C8L (Table 2).

Comparison pairs	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
Cycle 8 early vs. Cycle 0	0.0105	0.9634	0.6389	0.0096
Cycle 8 late vs. Cycle 0	0.7254	0.1242	0.329	0.9483
Cycle 8 late vs. Cycle 8 early	0.0034	0.1227	0.1571	0.007

Table 2: Statistical significance of individual haplotype frequencies at the *miR156* transcript region among all possible comparisons of C0, C8E, and C8L

Using two-way chi-square tests we found that the frequency of haplotype three at the *miR172* transcript region was significantly decreased by selection for late VPC ($p=0.0167$) and was significantly different between C8L and C8E ($p=0.0018$). The frequency of haplotype four at the *miR172* transcript region was significantly different between C8E and C8L ($p=0.000669$) (Table 3).

Comparison pairs	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4
Cycle 8 early vs. Cycle 0	0.4633	0.1145	0.5347	0.0592
Cycle 8 late vs. Cycle 0	0.8034	0.3597	0.0167	0.1209
Cycle 8 late vs. Cycle 8 early	0.3332	0.5177	0.0018	0.000669

Table 3: Statistical significance of individual haplotype frequencies at the *miR172* transcript region among all possible comparisons of C0, C8E, and C8L

Discussion:

Our analysis of sequence data 1900bps downstream from the *miR156* and 1500bps downstream from the *miR172* transcript regions reveals sequence variation within both regions. The variation in each region is organized into four distinct haplotypes that show a non-random response to selection for VPC. Haplotypes at the *miR172* transcript region significantly respond to selection for late VPC whereas haplotypes at the *miR156* transcript region significantly respond to selection for early VPC. These haplotypes may be linked to targets for selection in the pre-miRNA sequences or enhancer regions. These presumed targets may affect mature miRNA levels through various mechanisms. Potential mechanisms include: reduced DNA transcription at the *miR156* and *miR172* transcript regions, or reduced stability of *pre-miRNA156* and *pre-miRNA172* secondary structures. Although microRNA sequences are highly conserved, within species pre-miRNA polymorphisms have been found within populations of *Arabidopsis thaliana*. In *A. thaliana* these pre-miRNA polymorphisms alter pre-miRNA secondary structure and can reduce their stability thus reducing mature microRNA levels. (de Meaux et. al., 2008, Ehrenreich et. al., 2008). Polymorphisms in the *pre-miR156* and *pre-miR172* sequence may exist in maize and may be targets for selection linked to the haplotypes detected in this experiment. Interestingly, if selection reduced levels of *miR156* early VPC would be promoted while reduced levels of *miR172* would promote late VPC.

Conclusion:

Population Parentage:

Our parentage analysis of C0 did not succeed in identifying the parents of the population. Had we identified the parents we would have sequenced the parents at the *miR156* and *miR172* transcript regions to develop SNP markers. These SNP markers would have been used to screen

C0, C8E, and C8L for haplotype frequency changes between populations. With no parents identified, we detected the haplotype frequency changes using sequencing. Developing SNP markers from the C0 sequence data at the *miR156* and *miR172* transcript region would be useful to facilitate further studies of haplotype frequency changes at these regions in populations derived from C0.

Haplotype Frequency Analysis:

Our observation that haplotype frequencies at the *miR156* and *miR172* transcript regions significantly respond to selection for the timing of VPC supports the hypothesis that the variation at these regions is involved in the differential timing of VPC observed in C0, C8E, and C8L. These data contribute to the identification of important loci involved in VPC.

To validate the importance of variation at the *miR156* and *miR172* transcript regions in the response to selection for VPC intermediate cycles of selection should be haplotyped to determine whether there was a consistent response to selection. To investigate the genetic mechanism employed by the targets for selection in this experiment MIR156 and MIR172 levels in C0, C8E and C8L populations should be tested. If MIR156 and MIR172 levels differ, the stability of the pre-miRNA156 and pre-miRNA172 in should be investigated.

Understanding the genetic basis for VPC will facilitate maize breeding aimed at combining either early or late VPC with other desirable traits. Since early VPC in maize imparts partial resistance to common rust, incorporating early VPC into maize cultivars could reduce crop losses in Wisconsin where rust is prevalent.

References:

- Abedon, B. G., L. L. Darrah, and W. F. Tracy. 1996. *Corngrass1 (Cg1)* of maize (*Zea mays L.*) delays development of adult plant resistance to common rust (*Puccinia sorghi Schw.*) and European corn borer (*Ostrinia nubilalis Hubner*). *Journal of Heredity* 87:219-223.
- Basso, C. F., M. M. Hurkman, E. S. Riedeman, and W. F. Tracy. 2008. Divergent selection for vegetative phase change in maize and indirect effects on response to *Puccinia sorghi*. Accepted by Crop Science
- Chuck, G, AM Cigan, K Saeteurn, and S Hake. 2007. The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. *Nature Genetics* 39:544-549.
- Lauter N., A. Kampanl, M. Goebel, and S.P. Moose. 2005. *microRNA172* down-regulates *glossy15* to promote vegetative phase change in maize. *PNAS* 102:9412-9417.
- Poethig, R. S. 1990. Phase change and the regulation of shoot morphogenesis in plants. *Science* 250:923-930
- Riedeman, E.S., M.A. Chandler, and W.F. Tracy. 2008. Seven cycles of divergent recurrent selection for vegetative phase change and indirect effects on resistance to common rust (*Puccinia sorghi*) and European corn borer (*Ostrinia nubilalis*). Accepted by Crop Science.
- Rhoades, M. W., B. J. Reinhart, L. P. Lim, Burge, B. Bartel, and D. P. Bartel. 2002. Prediction of plant microRNA targets. *Cell* 110:513-320.
- Pataky, J. K., Pate, M. C., and Hulbert, S. H. 2001. Resistance genes in the *rp1* region of maize effective against *Puccinia sorghi* virulent on the *Rp1-D* gene in North America. *Plant Dis.* 85:165-168.