RAPD MARKER FREQUENCY CHANGES ASSOCIATED WITH SUGAR AND PIGMENT CONTENT IN TWO BEET POPULATIONS MODIFIED VIA RECURRENT SELECTION

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Abstract
Utility and extractability of betalain pigments from red beet is dependent upon native concentrations of both pigment and total dissolved solids (sugars). In an effort to develop red beet populations with elevated levels of betalain pigment and decreased levels of solids, recurrent half-sib family selection for high pigment and both high and low solids was practiced for seven cycles. This scheme resulted in the development of a high pigment-high solids and a high pigment-low solids population. Thirty-one RAPD primers were used to assess Random Amplified Polymorphic DNAs marker frequencies on genomic DNA samples isolated from 47 randomly-chosen individual plants in each of cycles 1, 3, and 6 in both populations. A total of 161 Random Amplified Polymorphic DNA markers were evaluated. Chi-square tests revealed a subset of markers which exhibited significant frequency changes across cycles and suggested these changes were associated primarily with selection. Selection was more effective in changing pigment concentration than solids content, however the two populations represent a 3% mean difference in solids values. Population-specific marker frequencies may indicate linkage of markers to genes controlling pigment or solids in red beet.

Introduction
Betalain pigments derived from red beet (*Beta vulgaris* L.) are used as colorants in a variety of foods (von Elbe et al. 1974; von Elbe and Maing 1973; Lauro 1991). During the past fifteen years, breeding efforts have been effective in increasing betalain pigment concentration in red beet (Wolyn and Gabelman 1986; Wolyn and Gabelman 1990), resulting in greater yields of pigment per unit area of beet. Inheritance of betalain pigment in red beet is likely controlled by both quantitative and qualitative factors (Pink 1993; Keller 1936; Wolyn and Gabelman 1986). The presence/absence of color is determined by two loci: *R* and *Y* (or *G*). Keller (1936) and Wolyn and Gabelman (1989) reported five alleles for *R*: *R*, R^t, *r*, R^p, and R^h, and three for *Y*: *Y*, Y^r, and *y*. In *R_Y_* genotypes, pigment concentration can be increased substantially with selection. A recurrent selection program initiated in 1978 increased betalain pigment concentration in two populations (Wolyn and Gabelman 1989). These data suggest pigment concentration responds to selection like a quantitative trait.

Dissolved solids, mostly sugars, present in beet extract limit production of highly concentrated pigment solutions for food dyes (Wolyn and Gabelman 1990). Divergent recurrent selection was used to develop high pigment-high solids (HPHS) germplasm for use in the beet canning industry and high pigment-low solids (HPLS) germplasm for extraction of natural colorants Wolyn and Gabelman (1990). At present eight cycles have been completed. Pigment concentration exhibited significant changes among cycles (Wolyn and Gabelman 1990; Breitbach and Goldman unpublished data). There has been no significant gain or loss in solids concentration over the cycles of selection for either population.

Molecular markers have provided a mechanisms for dentification of gene(s) controlling quantitative traits (Quantitative Trait Loci; QTL) in many plant species. Typically, molecular markers have been used to identify linkages to QTL through analysis of segregating progenies such as backcross or F_2 generations. Tracking molecular marker frequencies in breeding populations may also be used to identify QTL. This option has not been utilized as extensively as traditional backcross and F_2 methods, perhaps because of the lack of seed from cycles of populations under selection, or lack of experimental populations selected for the same trait over many generations. Furthermore, recombination between the marker locus and the QTL necessitates tight linkage for such

an approach to succeed. Despite these difficulties, a number of workers have used this approach for identiying molecular markers associated with quantitative traits such as yield and maize kernel chemical composition (Stuber et al. 1980; Stuber and Moll 1972; Kahler 1985; Sughroue and Rocheford 1994).

Knowledge of genetic changes at the molecular level during the recurrent selection program initiated by Wolyn and Gabelman would provide a better understanding of genetic control of pigment concentration and percent solids in *Beta vulgaris*. The objectives of this investigation were to 1) determine RAPD marker frequencies in two populations undergoing recurrent half-sib selection for pigment and solids concentration, and 2) determine whether marker frequency changes among cycles were due to selection or random genetic drift.

Materials and Methods

Half-sib recurrent selection for high pigment and both high and low solids was initiated in 1978 by J.F. Watson. Details of the recurrent selection scheme and pigment extraction are given in Wolyn and Gabelman (1990). Cycles 1, 3, and 6 (C1, C3, and C6, respectively) were chosen for investigation. Samples of 200 randomly selected seeds from each of the three cycles, 100 from each of the HPHS and HPLS populations, were sown in greenhouse flats. Ninety-four individuals were randomly selected from each cycle; 47 individuals from each of the HPHS and HPLS within a cycle. Leaf tissue was collected from 28 day old individual seedlings and frozen at -80°C. DNA was isolated from finely-ground, frozen leaf tissue using a modified CTAB method (Saghai-Maroof et al. 1984). A series of 200 arbitrary decamer oligonucleotide primers (Operon Technologies, sets AA-AI, Alameda, CA) were used to amplify genomic regions from individual beet plant samples via the polymerase chain reaction (Williams et al. 1990). These survey primers were screened for consistency of amplification and clarity. Thirty-one of the survey primers were chosen for further analysis of the cycles of selection. Reactions were run in 96-well Falcon assay plates according to protocols described in Paran et al. (1991). Each reaction contained 5 µl of DNA dilution (containing 5-10 ng of DNA), 12.8 µl of water, 2.5 µl of 10x buffer, 1.5 µl of 10 mM $MgCl_2$, 1.0 µl of 10 µM primer, 2.0 µl total of 1.25 mM each dNTP's and 0.2 µl of TAQ polymerase (2-5 units/µl). Two drops of mineral oil were added to assay plate wells to minimize evaporation. Controls containing no template DNA were added to each reaction plate. Amplification conditions were 1 minute at 94°C, 5 seconds at 94°C, 6 cycles of 30 seconds at 92°C, 1 minute at 36°C, 1 minute at 72°C and 36 cycles of 30 seconds at 92°C, 1 minute at 36°C and 1 minute at 72°C. Amplification reactions ran for 42 cycles in a MJ Research PTC-100 Programmable Thermal Controller. Amplification products were electrophoresed on 2% low EEO agarose gels. Bands were visualized via ethidium bromide staining. A 100 base pair ladder was used as a molecular weight standard on each gel. Amplification products in the form of fluorescent bands resulting from each primer-DNA combination were scored on a presence-absence basis. Scored amplification products ranged from 275-2000 base pairs. Amplification reactions yielded from 1-10 discreet, scorable amplification products, resulting in a total of 161 markers (Table 1). Amplification products were designated by primer name followed by molecular weight. Duplicate reactions were run throughout the experiment to check reproducibility of the amplification products obtained. Amplification products were only scored if they yielded good repeatability and clarity of bands over multiple amplifications. Thus, not all 282 individuals were scored for each primer/DNA interaction. Comparison of marker frequencies between populations within cycles were performed to determine whether RAPD marker frequencies changed significantly between HPHS and HPLS populations over different cycles. Comparisons of marker frequencies among cycles within populations were made to determine whether significant differences in marker frequencies resulted from the arecurrent selection process. Finally, evaluation of marker frequencies with significant trends among cycles within populations were made to determine if these changes were due to selection or drift (Shaffer et al., 1977).

Results and Discussion

Chi-square contingency tests at the $P<0.10$ level revealed significant marker frequency differences for 34 (21%) of the markers between HPHS and HPLS populations for C1

(Table 2). Twenty-eight (17%) significant marker frequency differences were detected between HPHS and HPLS populations for C3. Forty-seven (29%) significant marker differences between HPHS and HPLS populations were detected in C6.

Between HPHS and HPLS populations for C1, C3, and C6, both significant increases and decreases in RAPD marker frequency were detected. Markers AC01.800 and AC19.400 showed higher frequencies and greater increases in HPLS as compared to HPHS in all 3 cycles. More markers were significant for comparisons between populations in C6 than for either C1 or C3. Divergent selection in the HPHS and HPLS populations resulted in larger phenotypic differences between the two populations for pigment concentration the selection program proceeded. Despite the emphasis on selection for high pigment concentration in both populations, differences in total pigment concentration following six cycles of recurrent selection were noted; with higher concentrations present in HPHS. Reproductive isolation and genetic drift during the recurrent selection program coupled with continued phenotypic divergence between the two populations may be responsible for the presence of more significant marker loci differentiating HPHS and HPLS by C6.

Forty-two significant marker frequency changes were noted among cycles for HPHS. Fourty-four significant changes were noted among cycles for HPLS. Thirty-four, 28, and 47 significant difference sin marker frequency were detected between HPHS and HPLS in C1, C3, and C6, respectively (Table 2). Not all significant chi-square tests among cycles reflected linear trends in marker frequencies. Markers showing non-linearity were not evaluated further because they likely reflect random fluctuation in marker. Thirty eight of 44 significant marker frequency changes among cycles within HPLS exhibited linear trends. Twenty-five markers exhibited significant frequency increases, while 13 showed significant marker frequency decreases. Thirty-seven of the 38 markers which were significant and linear within HPLS were also significantly associated with selection.

Population-specific significance for certain primers was detected in among-cycle comparisons. Primers AA10, AA12, AC01, AC15, AE10, AG15, and AG17 were only found significant among cycles within HPLS. Primers AB01, AB11, AB15, AC20, AD04, AE02, AG15, and AI04 were only found significant among cycles within HPHS.

Markers exhibiting significant differences among cycles within populations or between populations within cycles may reflect linkage to genes controlling pigment concentration. Markers exhibiting frequencies that increase in either population (HPLS or HPHS) may be linked to genes controlling pigment concentration, however, this hypothesis does not take into account repulsion-phase linkages. Drift was only found in comparisons where significant effects due to selection were also present. This finding suggests marker frequency changes in the recurrent selection program were primarily due to the effects of selection rather than genetic drift.

A number of correlated traits change in response to selection for pigment concentration and percent solids. Pigment concentration is negatively correlated with root size (Nilsson 1973; Watson and Gabelman 1982), as is sugar concentration (Watson and Gabelman 1982; Powers 1957). Selection for high pigment concentration may have affected root size and alleles at corresponding loci associated with this trait may have changed in response to this selection pressure. The larger number of significant marker frequency differences observed in C6 as compared to C1 and C3 may additionally reflect linkage of these markers to genes controlling related traits such as root size. Stuber et al. (1980) pointed out that molecular marker loci initially in linkage disequilibrium with loci affected by selection should exhibit similar shifts in frequency. Because both populations were selected for high pigment concentration, the likelihood of similar shifts in marker frequencies in HPHS and HPLS would be increased.

Wolyn and Gabelman (1990) found directional but non-significant changes in percent solids in HPHS from cycles 1-3. We did not detect a significant difference among cycles of selection for percent solids in either HPHS or HPLS when remnant seed from each population and cycle was evaluated in a field experiment during 1994. Significant differences in percent solids among HPHS and HPLS were, however, observed following the synthesis of the cycle 1 populations (Wolyn and Gabelman, 1990). Over cycles of selection, HPHS exhibited a significantly higher percent solids than HPLS. Since the betalain pigment molecule requires a sugar molecule for formation (Clement et al. 1994), it

would follow that a correlated change for percent solids accompanied selection for elevated pigment concentration. HPHS and HPLS may therefore be treated as being selected for similar traits, given that both HPHS and HPLS exhibited increases in pigment concentration and no significant changes in percent solids over cycles. Detection of molecular markers associated with percent solids may require the development of populations divergently selected for solids without the additional selection pressure for pigment concentration.

Results from this investigation demonstrate the utility of screening for frequency changes in marker-linked regions of the red beet genome during recurrent selection for quantitative traits. A large number of significant marker frequency changes across cycles of selection within populations and between populations within cycles of selection were found in this investigation. Despite the large number of significant marker associations, relatively few exhibited significant linear changes across cycles of selection, suggesting many of these markers are either linked to genes not under selection or changing via random genetic drift of the red beet genome. Additionally, without a complete genetic map, it is impossible to assess whether these randomly-chosen primers are associated with priming sites well-spaced throughout the genome. Despite this limitation, the characterization of 161 amplification products provides for a substantial number of markers with which to evaluate changes in marker frequency in selected populations. Markers which exhibited significant linear changes in frequency among cycles of selection and which were shown to be due to the effects of selection may be candidates for marker-assisted recurrent selection programs designed to increase pigment concentration in red beet.

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Table 1. Operon primer number and sequence, number of samples amplified, number of amplification products, and number of amplification products scored for each primer.

primer number	primer sequence	no. samples amplified	no. amplification products	no. products scored
AA01	AGACGGCTCC	272	12	7
AA03	TTAGCGCCCC	252	15	8
AA10	TGGTCGGGTG	261	11	5
AA12	GGACCTCTTG	254	11	5
AA14	AACGGGCCAA	280	12	6
AB01	CCGTCGGTAG	264	6	3
AB09	GGGCGACTAC	261	12	9
AB11	GTGCGCAATG	277	8	5
AB14	AAGTGCGACC	254	11	7
AB15	CCTCCTTCTC	159	17	10
AB17	TCGCATCCAG	268	12	7
AC01	TCCCAGCAGA	219	11	5
AC06	CCAGAACGGA	267	19	10
AC15	TGCCGTGAGA	251	9	3
AC19	AGTCCGCCTG	262	11	3
AC20	ACGGAAGTGG	261	14	3
AD01	CAAAGGGCGG	259	15	7
AD02	CTGAACCGCT	267	9	3
AD04	GTAGGCCTCA	227	15	4
AD20	TCTTCGGAGG	282	4	1
AE02	TCGTTCACCC	268	10	2
AE07	GTGTCAGTGG	263	15	7
AE09	TGCCACGAGG	282	9	3
AE10	CTGAAGCGCA	264	13	4
AF05	CCCGATCAGA	274	13	6
AF11	ACTGGGCCTC	266	16	8
AF15	CACGAACCTC	267	8	1
AG15	CCCACACGCA	242	14	7
AG17	AGCGGAAGTG	279	8	5
AI04	CTATCCTGCC	260	11	5
AI17	CCTCACGTCC	269	6	3
Totals		282	357	161
Average		259	12	5

Table 2. Significance level and number of significant and non-significant chi-square values for obtained marker frequency changes and selection /drift calculations among cycles within HPHS and HPLS and between populations within a cycle.

Significance Level	Comparison within HPHS			within HPLS			Between		
	Among cycles	Selection	Drift	Among cycles	Selection	Drift	C1	C3	C6
*	14	10	5	12	17	5	11	6	11
**	16	13	1	19	19	3	17	13	20
***	12	19	2	13	18	0	6	9	16
ns	119	120	154	119	108	154	128	134	115

ns,*,**,*** denote not significant, and significance between 0.1 and 0.05, 0.05 and 0.01, and less than 0.01 probability levels, respectively