ASSESSING NOVEL GENES ASSOCIATED WTIH STARCH CONTENT IN MAIZE THROUGH TRANSCRIPT PROFILING ANALYSIS

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ABSTRACT

Presently there is increased interest in studying and improving crops for production of bio-fuels. Improving starch concentration in maize (*Zea mays* L.) would likely be a great asset for the ethanol industry. From the cross of the high starch maize line Illinois Low Protein cycle 90 (ILP^{90}) × B73 backcrossed to B73, 150 S_1, 138 S_3, and 138 S_3 testcross lines were developed. Transcript profiling analysis was employed to assess differences in gene expression between selected high and low starch S_1 families and testcross progenies. Two time points, 15 and 20 days after pollination (DAP), were evaluated. A model was implemented to identify overall differentially expressed genes in the two populations. In addition, the contrast of the 15 vs. 20 DAP was used to identify genes that increased or decreased relative expression during seed development. A total of 89 genes with consistent expression patterns across time points and populations were selected. None of the genes evaluated were found significant in the 15 vs. 20 DAP contrast indicating no major changes in gene expression between high and low families occur between the two time points. Only two known starch biosynthesis related genes were identified. One, alpha amylase inhibitor (AAI), showed the highest fold difference in expression among all genes represented on the chip. Further expression differential of this gene was evaluated in RT-PCR which strengthened the hypothesis of the gene being related to final starch concentration present in materials derived from ILP^{90}×B73. Our results strongly support a likely function of AAI gene in final starch accumulated in ILP^{90}×B73 derived materials. In order for higher starch concentration materials to serve as useful donors to commercial hybrids, they should provide reasonably competitive grain yields. Thus in the oral presentation we provide information on yield trial performance of testcross hybrids of ILP/B73 derived materials in relation to commercial checks and experimental hybrids derived from PVP released materials.

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INTRODUCTION

Improving and modifying field crops to respond to the needs of society and increasing populations has, and continues to be, a major goal in plant breeding. Breeding in combination with various genetic tools is routinely implemented to improve key traits in field crops. However, according to FAO (http://www.fao.org), by 2030 hundreds of millions of people will remain hungry. Added to the responsibility and challenges of providing enough food for the growing worldwide population, plant breeders are increasingly facing other demands to improve crops for diverse and new end uses. Presently the improvement of crops for production of bio-fuels is receiving considerable attention. However, breeding for higher yields continues to be of paramount importance, regardless of end use.

Cereal grains provide the greatest contribution of energy in the human diet, as starch is the major caloric source, comprising 55 to 75% of daily human calorie intake (Pan 2000). Maize (*Zea mays* L.) is the highest yielding grain crop in the world, and is one of the major sources of starch for both food and industry products. Thus increasing starch in maize grain is a relevant breeding objective for more than one reason. Recently there has been considerable interest in the use of association analysis to identify favorable alleles of genes that can be used to accelerate selection (Zhu et al., 2008). The structural genes in the starch synthetic pathway in maize are well characterized (Boyer and Hannah 2001). However recent association analysis studies have shown a lack of genetic diversity in these genes which suggests that major increments in maize starch concentration likely will not come from selection of alleles of pathway genes (Whitt et al. 2002). Therefore, the identification of genes that affect or interact with the pathway, or have a regulatory function, and thus could have an impact on controlling amounts of starch produced by the plant is a logical step towards increasing starch concentration.

A significant change in kernel composition traits in maize was shown to be possible through divergent selection by the Illinois Long-term Selection Experiment (ILTSE). The experiment showed that selection can greatly increase or decrease protein and oil in maize, and also showed that changing one of the kernel components led to change in other major kernel components (Dudley and Lambert 2004). One such example was selection for low protein in the Illinois Low protein (ILP) strain. During selection for low protein, starch was greatly increased resulting in a strain having the highest amount of starch ever achieved in maize grain (~75%). Thus, the molecular and genetic characterization of ILP is of great interest as it could reveal novel alleles of genes affecting starch concentration in maize that could easily be transferred into elite inbred lines to produce hybrids with increased starch.

The ILTSE experiment has been ongoing for over 100 years. Limits to low oil and low protein were reached, but thus far limits to selection for high oil or high protein have not been observed (Dudley 2007). Overall, progress from selection was much greater than could have been predicted. Dudley (1977) proposed that the presence of large numbers of loci with low frequency of favorable alleles in the original population could have accounted for the progress from selection. This hypothesis has been largely supported by a number of studies involving the ILTSE strains which revealed a large number of quantitative trait loci (QTL) involved in the control of starch, protein, and oil (Dudley et al. 2004; Laurie et al. 2004; Clark et al. 2006; Willmot et al. 2006; Dudley et al. 2007). Other studies suggested the importance of epistatic effects and favorable mutations that could have occurred during cycles of selection (Goodnight 2004; Eitan and Soller 2004; and Walsh 2004). Recently, the observation of more epistatic

interactions than expected by chance in Illinois High Oil (IHO) × Illinois Low Oil (ILO) and Illinois High Protein (IHP) × ILP populations suggested epistasis to be a factor in the continued response to selection in Illinois long-term selection strains (Dudley 2008). Progress from selection may have been a function of several combined factors, including contributions from major QTL. With current molecular technology, it is possible to further characterize these strains in order to identify genes that could have had a significant role in quantitatively influencing the biosynthetic pathways responsible for protein, starch, and oil.

Microarray data analysis is a very powerful tool for identifying candidate genes that are associated with control of metabolic, molecular and cellular functions in different tissues (Hoheisel 2006). Microarray technology has become a routine laboratory tool for studying changes in expression of a large number of genes in parallel, and the resulting information serves as an important tool in functional genomics. The expression profiles of genes with no known function have also been useful for assigning putative roles for some genes in the category.

In this study we assessed differences in gene expression of higher and lower starch concentration progenies derived from the cross of Illinois Low Protein cycle 90 (ILP^{90}) to B73, backcrossed to B73. Hand pollinated ears were sampled at different stages of development from selected high and low starch families and assayed through microarray protocols. The results provide information on genes controlling starch concentration in ILP^{90}×B73 derived materials, and also provide suggestions for genes that may have been key factors in the response to selection for low protein/high starch in ILP.

In the oral portion of the presentation we will provide information on yield trial performance of testcrosses of ILP/B73 derived materials. These results will be presented in relation to contemporary commercial hybrid check performance, and also in relation to experimental hybrids derived from F3s of crosses of PVP released materials. This information will be provided because in order for higher starch concentration materials to more likely serve as potential useful donors, they should provide reasonably competitive grain yields.

MATERIALS AND METHODS

Plant Materials: The maize inbred B73 was crossed by a single plant from ILP^{90}. A single F_1 plant was then backcrossed to B73, and the backcross (BC_1) progeny were self-pollinated to produce a population of 150 (ILP^{90}×B73)$B73S_1$ lines. The (ILP^{90}×B73)$B73S_1$ plants were advanced by single seed descent to develop (ILP^{90}×B73)$B73S_3$ lines. Only 138 (ILP^{90}×B73)$B73S_3$ lines were obtained, as some lines failed to produce ears with seed set. The (ILP^{90}×B73)$B73S_3$ lines were crossed to an Illinois Foundation Seeds tester (Fr616) to produce a testcross population consisting of 138 testcross lines, [(ILP^{90}×B73)$B73S_3$]Fr616.

Field Evaluations: The plant materials were grown at the University of Illinois Crop Sciences Research and Education Center at Urbana, Illinois. The 150 (ILP^{90}×B73)$B73S_1$ lines were grown along with the parents in two replications in 1993, 1994, 2003, 2004, and 2005. The 138 (ILP^{90}×B73)$B73S_3$ lines were grown in two replicates in 2002 and 2005. The 138

[(ILP^{90}×B73)B73S$_3$]Fr616 testcrosses were grown in three replications in one environment in 1999, three replications in each of two environments in 2001, and three replications in one environment in 2002. In all experiments, entries in each replicate were randomized in an incomplete block alpha (0,1) design.

Phenotypic Evaluations and Selection: For each replication of the (ILP^{90}×B73)B73S$_1$ and S$_3$ populations, a balanced bulk of seed was made from several self-pollinated ears within each row. For the [(ILP^{90}×B73)B73S$_3$]Fr616 population, a seed sample from each plot was collected from the combine at harvest. Approximately 50g of each seed sample was ground in an M-2 Stein Mill for 90 seconds. Ground samples were thoroughly mixed, and starch was measured using a Dickey-john GAC III near-infrared reflectance (NIR) instrument according to well-established procedures (Hymowitz, et al., 1974). Estimates of starch concentration are expressed on a percentage basis and hereafter are referred to as simply starch.
For each of the populations analyzed, best linear unbiased predictors (BLUPs) for each line were calculated using the model: $y_{ijkl} = \mu + \alpha_i + \beta_{j(i)} + \lambda_{k(ij)} + \delta_l + \alpha\delta_{il} + \varepsilon_{ijkl}$, where y represents the observed value for starch in each genotype, α_i is the effect of the i^{th} year, $\beta_{j(i)}$ is the effect of the j^{th} replication within the i^{th} year, $\lambda_{k(ij)}$ is the effect of the k^{th} block in the j^{th} replication of the i^{th} year, δ_l is the effect of the l^{th} genotype, $\alpha\delta_{il}$ is the interaction effect of the i^{th} year with the l^{th} genotype, and ε_{ijkl} represents the residual error. All the effects in the model were considered random, and were derived using SAS PROC MIXED in the statistical software package. The highest and lowest four families for starch were then selected in each population for further analysis.

Sampling: Selected high and low starch families from each of the three populations, ((ILP90×B73)B73S1, S3, and [(ILP^{90}×B73)B73S$_3$]Fr616), based on data available at the time, were grown and self pollinated in two replicates in the summer of 2005. Ears were collected at 10, 15, and 20 days after pollination (DAP) and frozen under liquid nitrogen. Kernels were separated from cobs while the ears were still frozen and kept at -80^{o}C until further analysis. Remnant pollinated ears from each line were left in the field until ready to harvest, and starch was estimated using NIR.

Whole-genome expression analysis: Sampled kernels were sent to CERES, Inc., (Thousand Oaks, CA) where RNA was extracted from bulks of kernels from the four high and four low starch lines for each of (ILP^{90}×B73)B73S$_1$ and [(ILP^{90}×B73)B73S$_3$]Fr616 populations. The procedure consisted of bulking the four high and four low starch lines for each rep within each population and extract RNA from bulked samples. The Agilent 44k two channel oligo microarray platform was implemented for assessing gene expression profile differences. The experiment comprised of two replicates, each with reciprocal dye swap for each time point assessed in the two populations. Two time points, (15 and 20 DAP), were assayed on the (ILP^{90}×B73)B73S$_1$ population, and three time points (10, 15, and 20 DAP) were assayed on the [(ILP^{90}×B73)B73S$_3$]Fr616 population.
Expression data was preprocessed by the Agilent G2567AA Feature Extraction Software (v8.1). Quality of the preprocessed data was assessed by evaluation of box plots and MA plots for each slide. MA plots are used to visualize intensity-dependent ratio of raw microarray data where M and A are defined as: $M = \log_2 R - \log_2 G$, $A = \frac{1}{2}\left(\log_2 R + \log_2 G\right)$ where R is the red and G is

the green dye expression intensity, respectively. The 10 DAP time point was assessed with microarrays for only the [(ILP^{90}×B73)B73S$_3$] Fr616 population, making the experimental design unbalanced. Furthermore, starch is not accumulated at a significant rate at this time point and thus, only the 15 and 20 DAP time points were considered in the analysis presented here. Microarray data analysis for the two populations assayed was done in SAS PROC GLM using the model $y_{ijkl} = \mu + \alpha_i + \beta_j + \lambda_k + \delta_l + \varepsilon_{ijkl}$ where, y is the $\log_2$ of the ratio of high vs. low starch normalized expression, μ is the mean log ratio, α_i is the effect of the i^{th} replication, β_j is the j^{th} dye swap effect, λ_k is the k^{th} DAP effect, δ_l is the effect of the l^{th} population, and ε_{ijkl} is the random error term. All possible interactions among terms in the model were first tested but were not significant, and thus were not included in the final model. Log_2 ratio of high to low starch samples means were estimated for each gene, and 15 vs. 20 DAP contrasts between $\log_2$ ratios were also calculated. The use of contrasts enables the identification of genes that increase or decrease transcription during seed development. Differentially expressed genes were identified by testing the deviation of the $\log_2$ ratio mean (μ) from zero. False Discovery Rate (FDR) was used to correct raw p-values for multiple testing (Benjamini and Hochberg, 1995). FDR was calculated using the PROC MULTTEST procedure in SAS and values less than 0.01 were declared significant.

Cluster Analysis: Hierarchical clustering analysis was implemented to group significantly differentially expressed genes. Clustering was done by calculating Euclidean distances between mean estimates of normalized $\log_2$ ratios across reps and dye swaps for each time point in each population. The average distance method was used to produce hierarchical clusters. Cluster analysis, along with an expression heatmap, was performed using the Pattern Recognition and Data Mining in Microarray Analysis (*tightClust*) software (Tseng and Wong 2005). The heatmap shows the expression pattern of each gene across time points and populations (red: up-regulated, green: down-regulated, black: value close to zero) (Fig. 1). Therefore, and since we are evaluating the ratio of high to low starch, a value greater than zero implies up-regulation of a gene in the high starch lines and a negative value implies down-regulation.

Real-Time Quantitative PCR: Total RNA was extracted from 50mg of bulks from ground kernels of high and low starch families of (ILP^{90}×B73)B73S$_1$, (ILP^{90}×B73)B73S$_3$, and [(ILP^{90}×B73)B73S$_3$]Fr616 populations using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For the (ILP^{90}×B73)B73S$_1$ and (ILP^{90}×B73)B73S$_3$ high and low starch samples, two biological replicates were used. One biological replicate was sampled for [(ILP^{90}×B73)B73S$_3$]Fr616 population. 5ug of total RNA was treated with turbo DNAse (Applied Biosystems, Foster City, CA) to eliminate potential genomic DNA contamination. From DNAse treated samples, approximately 3ug of total RNA was reverse transcribed (RT) using SuperScript^{TM} First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To minimize variations, all RNA samples were reverse-transcribed simultaneously. Sample cDNA was then extracted using a standard phenol-chloroform (1:1) protocol. All cDNA samples were quantified using the Nanodrop spectrophotometer (Nanodrop Technologies). Triplicate quantitative assays were performed with an ABI PRISM 7000 Sequence Detection System using the Power SYBR Green PCR Master Mix kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Several negative control reactions, (without RT), were prepared and analyzed in parallel with the unknown samples. Gene-specific primers were designed using Integrated DNA Technologies

(IDT) RT-PCR online tools. The relative quantification method ($2^{-\Delta\Delta Ct}$) was used to evaluate quantitative variation between samples examined according to Livak and Schmittgen (2001). RT-PCR data was imported and analyzed in Microsoft Excel.

An alpha amylase inhibitor gene (AAI) (ID: 4009913) was selected for further validation with quantitative RT-PCR. An RT-PCR marker was designed based on the cDNA sequence for AAI provided by Agilent with primer pair sequences 5'AATCCGTCGTTGTTCCTTCAGCTC (sense) and 5'TGAGGGTCATGATGGACGGTATGC (antisense). An ubiquitin gene with primer pair sequences 5'GTCATGGGTCGTTTAAGCTGCCGAT (sense) and 5'GCACACACA ACACAACCGGTCCAT (antisense) was selected as a reference. For the $2^{-\Delta\Delta Ct}$ to be valid, the amplification efficiencies of the target and reference gene must be approximately equal. In order to determine whether target and reference gene had the same amplification efficiency 50, 10, 5, 1, 0.5, and 0.1 ng/ul of cDNA were RT-PCR assayed for both genes in triplicates. The average Ct (Ct, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold) was calculated for each dilution for both genes and the ΔCt (Ct, target – Ct, reference) was determined. Regression of the ΔCt on the log cDNA dilution was computed in SAS PROC REG to determine the slope. If the absolute value of the slope is close to zero, the efficiencies of the target and reference genes are similar; and thus, the ΔΔCt calculation for the relative quantification of target may be used (Livak and Schmittgen, 2001). The estimated slope of the line was 0.026, meeting the assumption of similarity. Consequently, the ΔΔCt method was used to analyze the data.

RESULTS

Phenotypic Evaluations of Selected Lines: The four high and low starch families used for microarray analysis were selected based on starch concentration values over several years and environments. However, as starch concentration is under polygenic control, and the trait is affected by the environment, differences in starch observed in previous years/environments may or may not correlate with the actual differences measured at gene expression level for the 2005 environment. Therefore, for the three populations ($(ILP^{90}\times B73)B73S_1$, S_3 and $[(ILP^{90}\times B73)B73S_3]Fr616$, estimates of starch concentration were obtained in remnant pollinated ears harvested from the same rows from which developing ear samples were collected for microarray analysis. The difference in starch between high and low starch families evaluated was the greatest for the $(ILP^{90}\times B73)B73S_3$ population (33 gKg^{-1}) (Table 1). The $(ILP^{90}\times B73)B73S_1$ and $[(ILP^{90}\times B73)B73S_3]Fr616$ populations showed smaller differences in starch for selected lines, 18 and 15 gKg^{-1}, respectively (Table 1).

Microarray data Analysis: MA and box-plots for the preprocessed microarray data indicated the data was well normalized (data not shown). Thus, no further normalization was done prior to subsequent analysis. A total of 345 genes were differentially expressed for the $(ILP^{90}\times B73)B73S_1$ and $[(ILP^{90}\times B73)B73S_3]Fr616$ populations. These 345 genes were identified by testing the deviation of the $\log_2$ ratio mean (μ) from zero using the model described in materials and methods. Contrasts comparing differentially expressed genes for the 20 vs. 15 DAP did not reveal any significant changes in the pattern of differences in expression over the

evaluated time points. From the 345 differentially expressed genes, a subset that was considered more relevant and reliable was selected based on expression patterns in the two populations. We considered a gene to be more relevant and reliable if it essentially showed the same expression pattern across the two times points in both populations. Selection was done visually and only genes that showed either a positive or a negative $\log_2$ ratio for all time points and populations was considered. This process resulted in identification of a set of 89 differentially expressed genes for further analysis (Table 2, Fig. 1).

Cluster Analysis: Cluster analysis was performed on the 89 selected genes in order to group genes with similar expression changes between high and low starch at different time points in the different populations. Several clusters were evident. The heatmap indicated that there were similar numbers of up and down-regulated genes, 45 and 44, respectively (Fig. 1). The principal array clusters did not feature any clear discriminator between time points in the two populations (Fig. 1). This result was somewhat expected as only genes with consistent expression patterns across time points and populations were considered. Two genes did not group with any others (Fig. 1). The unclustered genes were AAI (ID: 4009913) and a disease resistance response protein-like protein (ID: 4003259), and both were up-regulated in high starch lines (Fig. 1).

Real-Time Quantitative PCR: AAI gene was selected for validation with quantitative RT-PCR. The gene was selected first, because it is known to indirectly affect starch (Franco et al. 2002); and second, because it showed, by far, the greatest overall fold change (11.3) (Table 2). Overall fold change here pertains to the average fold change estimated across time points and populations. Although microarray analysis was performed on high and low starch samples on only two of the populations: $(ILP^{90}\times B73)B73S_1$ and $[(ILP^{90}\times B73)B73S_3]Fr616$; RT-PCR quantification for the AAI gene was assessed on high and low starch samples of all the three populations: $(ILP^{90}\times B73)B73S_1$, $(ILP^{90}\times B73)B73S_3$, and $[(ILP^{90}\times B73)B73S_3]Fr616$. Measuring expression of the gene in a population not assayed with microarrays can provide further validation of gene effects in $ILP^{90}\times B73$ derived materials. For the $(ILP^{90}\times B73)B73S_1$ population sampled at 15 DAP, AAI was not significantly differentially expressed in high vs. low starch samples, however, a 17.4 fold difference was measured at 20 DAP (Table 3). In the $(ILP^{90}\times B73)B73S_3$ population, RT-PCR analysis revealed a 3.1 fold differential at 15 DAP and almost a 57 fold increase at 20 DAP (Table 3). For the $[(ILP^{90}\times B73)B73S_3]Fr616$ population, a 10 fold change was observed at 15 DAP and a 1.3 fold change at 20 DAP (Table 3). Overall, RT-PCR confirmed the expression pattern of AAI gene, which suggests that the gene may affect starch concentration in $ILP^{90}\times B73$ materials.

DISCUSSION

Microarray experiments generally yield enormous amounts of data that need to be adjusted for various sources of variability in order to identify important genes among the many that are measured. Given the inherent ambiguity in microarray data analysis, combined analysis of multiple populations may yield useful and insightful results. If a gene is significantly differentially expressed in more than one population and in the same direction, there is more confidence in relating this gene to the treatment under evaluation. We took advantage of

assaying expression analysis in high and low starch families of two related populations to more reliably select relevant genes. Furthermore, genes that did not show a consistent expression pattern across time points and populations evaluated were excluded from this analysis. By doing this we do not imply lack of relevance of the removed genes, however this procedure seemed appropriate for selecting a subset of the likely most relevant ones.

A total of 89 differentially expressed genes showing consistent expression patterns underwent detailed analysis (Table 2, Fig. 1). From the 89 selected genes, only 39 have a known function. Several structural genes affecting starch synthesis/degradation were represented on the chip. However, only one (putative 4-alpha-glucanotransferase, ID: 4002443) was found to be significantly differentially expressed. This gene codes for an enzyme (EC 2.4.1.25) that catalyzes a chemical reaction that transfers a segment of a 1,4-alpha-D-glucan to a new position in an acceptor carbohydrate, which may be glucose or a 1,4-alpha-D-glucan. The observation that only one biosynthetic gene was differentially expressed may suggest that differential starch accumulation in the ILP^{90}×B73 materials may be largely due to genes other than known structural genes.

Of the selected genes with known function, only two appear related to starch synthesis/degradation: putative 4-alpha-glucanotransferase (ID: 4002443) and an AAI (ID: 4009913). Both genes were consistently up-regulated in the higher starch lines, though 4-alpha-glucanotransferase differential expression was not as clear cut as with AAI. The AAI gene showed a fold change of 11.3 whereas 4-alpha-glucanotransferase showed only about one fold change in expression (Table 2). Thus, only alpha amylase inhibitor presented clear, strong evidence for a potential role in starch accumulation in ILP^{90}×B73 materials based on expression analysis.

Among other known genes that were differentially expressed, there were two genes related to fatty acid composition. The two were classified as lipoxygenases (ID: 3988223, ID: 4002475) which are enzymes that affect fatty acid composition. Products of lipoxygenases are involved in diverse cell functions. These two genes were consistently down-regulated in higher starch lines (Table 2). The relative levels of the major kernel components starch, oil and protein are correlated with changes in each of these kernel components associated with changes in the other kernel components (Dudley and Lambert, 1992). For example, higher starch levels are associated with lower oil levels. Thus, finding genes affecting fatty acid composition expressed at lower levels in the higher starch families is potentially consistent with higher levels of starch and corresponding lower levels of oil.

Genes of unknown function which have similar expression profiles to genes known to be involved in the starch synthesis pathway may also function in the pathway. While AAI did not cluster with any other genes (Fig. 1), two genes with unknown function (ID: 3993572, ID: 4002778) grouped closely with putative 4-alpha-glucanotransferase (ID: 4002443). Interestingly, in the same cluster there was a putative nucleotide DNA-binding-like protein (ID: 3996734). Such genes form candidates for future further analysis.

The starch in maize is composed of two glucose polymers: amylose, that is predominantly made of linear chains of α1,4 linked glucose residues that adopt a helical configuration within the granule; and amylopectin, a highly branched glucan with α1,4 glucose units linked by α1,6 glycosidic bonds that form insoluble, semicrystalline granules. Our analysis

suggests that the AAI gene contributes to differences in starch in ILP^{90}×B73 materials. Alpha amylases are enzymes that help digest/degrade starch in most living organisms through breakage of the α-1,4 linkages. The function of alpha amylase inhibitors (AAIs) as, the name indicates, is to impede the function of alpha amylases so that starch is not degraded, or more likely is degraded at a slower rate. The activity of members of the AAI gene family has been reported in many plant species such as barley, rice, wheat, maize, finger millet, and jobi tear's seeds (Abe et al., 1993; Yamagata et al., 1998; Ohtsubo and Richardson 1992; Gvozdeva et al., 1993; Zemke et al., 1991; Blanco-Labra and Iturbe-Chinas 1980; Strobl et al., 1998; Ary et al., 1989). Most of the reports describe the gene acting on alpha amylases of insect pests, slowing or even stopping completely the digestion of plant starch by insect gut digestive alpha amylases. However, the interaction of AAIs with endogenous alpha amylases is not well understood. Our results suggest that an AAI may be acting on endogenous alpha amylases to affect higher final starch content and, further investigation of this hypothesis is required.

The expression patterns of the AAI gene were further validated through RT-PCR. Results were in overall agreement with microarray data analysis. Both microarray and RT-PCR data analysis indicated slight differences in the expression pattern among populations. From microarray analysis, the expression differential of the gene appeared to increase from 15 to 20 DAP in $(ILP^{90}×B73)B73S_1$ whereas in the testcross population expression differential seemed consistently high across the two time points. The trend observed in microarrays for $(ILP^{90}×B73)B73S_1$ was very similar to the results found with RT-PCR for this population however for the testcross population, a much smaller expression differential was measured by RT-PCR for the 20 DAP time point when compared with the measured by microarrays. This result could be due to sampling and the fact that only one biological replicate was used for evaluating gene expression in this population. The greatest fold change in expression was observed for the $(ILP^{90}×B73)B73S_3$ population at 20 DAP (~57 fold) (Table 3). Differences in starch concentration between high and low starch selected lines were the greatest for this population (33 gKg^{-1}). The selected lines from this population are more homozygous for all genes, likely including AAI (Table 1). This observation may indicate that greater differences in final starch concentrations observed between lines may be related to greater expression differences of the gene. The finding of a difference in gene expression of this gene observed in another related population not assayed in the arrays further supports its potential importance to differences in starch accumulation among these maize lines.

CONCLUSIONS

Transcript profiling analysis was performed to investigate gene expression differences in selected high and low starch families from ILP^{90}×B73 backcross to B73 derived populations. Three populations were derived from this backcross, two per se and one testcross. Microarray data analysis in two populations, one per se and the testcross, revealed 89 consistently differentially expressed genes. Two of the selected genes were related to starch: AAI and 4-alpha-glucanotransferase. AAI gene showed, the greatest fold difference in expression, and is also logically related to starch levels and therefore was selected for further validation through quantitative RT-PCR. Evaluation of expression of the gene measured with RT-PCR was performed in the two populations assessed in the microarrays plus the S_3 per se population not assayed in the microarrays. RT-PCR results confirmed the microarray results and the gene was hypothesized to be related to final starch concentration in materials derived from ILP^{90}×B73. Fold change differences of the gene were larger in materials with greater differences in starch concentration, which strengthened this hypothesis. We find AAI to be a primary candidate for an effect on final starch accumulated in ILP^{90}×B73 derived materials, however the other identified genes certainly may also contribute to starch levels.

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TABLES AND FIGURES

Table 1. Mean starch content in the four selected high and low starch lines from (ILP^{90}×B73)B73S$_1$, (ILP^{90}×B73)B73S$_3$, and [(ILP^{90}×B73)B73S$_3$]Fr616 populations grown in 2005.

Mean Starch (gKg^{-1})	
High Starch Lines	**Low Starch Lines**
(ILP^{90}xB73)B73S$_1$	
711 ± 0.28*	693 ± 0.60
(ILP^{90}xB73)B73S$_3$	
710 ± 0.21	677 ± 0.53
[(ILP^{90}xB73)B73S$_3$]Fr616	
698 ± 0.25	683 ± 0.25

*Standard errors attached.

Table 2. Consistently differentially expressed genes in high vs. low starch samples from $(ILP^{90}\times B73)B73S_1$ and $[(ILP^{90}\times B73)B73S_3]$Fr616 populations.

gene ID	Description of related protein	FC*	tValue	FDR
3973112	putative 60S ribosomal protein L31 [Oryza sativa (japonica cultivar-group)] >gi|45735864|dbj|BAD12898.1|	-1.1	-15.2	0.00005
3973423	putative RNA polymerase III [Oryza sativa (japonica cultivar-group)] >gi|29788820|gb|AAP03366.1|	-2.0	-7.6	0.00319
3973908		1.6	7.8	0.00265
3973957	Putative phosphoserine aminotransferase [Oryza sativa (japonica cultivar-group)] >gi|21397263|gb|AAM51827.1|	1.1	6.1	0.00938
3974264		-1.1	-6.5	0.00699
3975143		1.4	8.0	0.00240
3975178	unknown protein [Oryza sativa (japonica cultivar-group)] >gi|23237902|dbj|BAC16476.1|	-1.1	-8.3	0.00174
3975310		-1.9	-8.8	0.00129
3975464	putative peptidyl-prolyl cis-trans isomerase, chloroplast precursor [Oryza sativa (japonica cultivar-group)] >gi|13486733|dbj|BAB39968.1|	2.1	6.1	0.00938
3975813	PREDICTED P0594D10.136 gene product [Oryza sativa (japonica cultivar-group)] >gi|34906844|ref|NP_914769.1| putative phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplast precursor [Oryza sativa (japonica cultivar-group)]	-1.1	-14.8	0.00005
3976688	PLASMA MEMBRANE ATPASE 2 (PROTON PUMP 2)	-1.6	-7.8	0.00273
3976960	unknown protein [Oryza sativa (japonica cultivar-group)] >gi|33146618|dbj|BAC79906.1|	-1.5	-12.2	0.00016
3977706	F3F9.7 [Arabidopsis thaliana]	-1.2	-6.2	0.00873
3978786	HUELLENLOS-like protein [Oryza sativa]	2.2	16.0	0.00004
3979424	putative dihydrokaempferol 4-reductase [Oryza sativa (japonica cultivar-group)] >gi|33146535|dbj|BAC79712.1| putative NADPH HC toxin reductase [Oryza sativa (japonica cultivar-group)] [Oryza sativa (japonica cultivar-group)]	-1.9	-9.7	0.00074
3979630	P0010B10.2 [Oryza sativa (japonica cultivar-group)]	1.5	7.4	0.00350
3980361	putative senescence-associated protein [Oryza sativa (japonica cultivar-group)] >gi|42407435|dbj|BAD10042.1|	-1.4	-6.7	0.00615
3981125		1.2	11.1	0.00030
3982778		-1.4	-7.8	0.00273
3983220	putative ankyrin-kinase [Oryza sativa (japonica cultivar-group)] >gi|33146898|dbj|BAC79897.1|	1.6	12.9	0.00013

Table 2. Continued.

gene ID	Description of related protein	FC	tValue	FDR
3983388	putative 26S proteasome non-ATPase regulatory subunit 3 [Oryza sativa (japonica cultivar-group)] >gi|50725298|dbj|BAD34300.1| putative nuclear antigen 21D7 [Oryza sativa (japonica (japonica cultivar-group)]	2.4	7.4	0.00366
3983432	putative blue copper binding protein [Oryza sativa (japonica cultivar-group)] >gi|38636760|dbj|BAD03003.1|	1.3	7.7	0.00282
3983686	PREDICTED OJ1003_B06.33 gene product [Oryza sativa (japonica cultivar-group)] >gi|50911415|ref|XP_467115.1| putative protein kinase [Oryza sativa (japonica cultivar-group)] >gi|49388553|dbj|BAD25672.1|	-1.4	-6.6	0.00666
3984008	glycosyl hydrolase family 17-like protein [Oryza sativa (japonica cultivar-group)] >gi|22775658|dbj|BAC15512.1|	1.2	6.2	0.00879
3984235	ribosomal protein S11 [Zea mays] >gi|82722|pir||S16577 ribosomal protein S11 - maize >gi|133867|sp|P25460|RS11_MAIZE 40S RIBOSOMAL PROTEIN S11	1.3	6.3	0.00812
3984461	putative hypersensitivity-related (hsr)protein [Oryza sativa (japonica cultivar-group)] gi|31432925|gb|AAP54496.1|	-1.2	-6.8	0.00575
3985130		-1.4	-8.3	0.00174
3985683	putative protein phosphatase [Oryza sativa (japonica cultivar-group)] >gi|9909177|dbj|BAB12036.1|	-1.7	-6.9	0.00542
3986053	putative 40S ribosomal protein [Arabidopsis thaliana] >gi|15028253|gb|AAK76715.1| putative 40S ribosomal protein; contains C-terminal domain [Arabidopsis thaliana] >gi|4582468|gb|AAD24852.1|	-1.1	-6.2	0.00921
3986162	calcium binding EGF domain containing protein [Oryza sativa (japonica cultivar-group)]	-1.7	-8.8	0.00127
3986946		1.2	6.4	0.00757
3987546	hypothetical protein AdehDRAFT_1030 [Anaeromyxobacter dehalogenans 2CP-C] >gi|66857206|ref|ZP_00401261.1|	3.7	6.7	0.00629
3987702	PREDICTED OJ1148_D05.1 gene product [Oryza sativa (japonica cultivar-group)] >gi|50911659|ref|XP_467237.1| putative silverleaf whitefly-induced protein 1 [Oryza sativa (japonica cultivar-group)]	2.1	9.4	0.00086
3987918	COP1-interacting protein 7 (CIP7)-like protein [Oryza sativa (japonica cultivar-group)] >gi|33146927|dbj|BAC79948.1|	3.4	9.7	0.00075
3988223	lipoxygenase [Zea mays]	-3.5	-10.3	0.00053
3988267	unknown protein [Oryza sativa (japonica cultivar-group)] gi|49387643|dbj|BAD25837.1|	-1.1	-6.5	0.00699

Table 2. Continued.

gene ID	Description of related protein	FC	tValue	FDR
3988527		-1.6	-7.9	0.00242
3988599		2.3	14.6	0.00005
3989248	P0489A01.12 [Oryza sativa (japonica cultivar-group)] >gi|9081782|dbj|BAA99521.1|	1.2	7.5	0.00335
3989637		1.3	6.9	0.00547
3990090		-1.1	-6.7	0.00638
3990105	unknown protein [Oryza sativa (japonica cultivar-group)]	1.2	6.6	0.00666
3990413		-1.6	-9.5	0.00082
3990554	putative translation initiation factor IF-2 [Streptomyces avermitilis MA-4680] >gi|39931258|sp|Q82K53|	-1.8	-6.4	0.00749
3991208		-1.1	-8.2	0.00198
3992450		1.2	10.2	0.00053
3992917		-1.9	-7.6	0.00319
3993097		1.4	9.1	0.00110
3993416	putative coatomer protein complex, beta prime subunit [Oryza sativa (japonica cultivar-group)]	2.0	6.6	0.00666
3993572	unnamed protein product [Oryza sativa (japonica cultivar-group)] >gi|7340858|dbj|BAA92948.1|	1.2	9.1	0.00110
3993601		-1.6	-6.1	0.00970
3993708	glyoxalase family-like protein [Oryza sativa (japonica cultivar-group)] >gi|34395261|dbj|BAC83945.1|	-1.6	-6.7	0.00638
3994103	unnamed protein product [Oryza sativa (japonica cultivar-group)] >gi|6539559|dbj|BAA88176.1|	-1.2	-11.4	0.00025
3995565	unknown protein [Arabidopsis thaliana] >gi|14334574|gb|AAK59466.1| unknown protein [Arabidopsis thaliana] >gi|51970916|dbj|BAD44150.1| prolyl carboxypeptidase like protein [Arabidopsis thaliana] >gi|51970726|dbj|BAD44055.1|	-1.0	-6.6	0.00690
3995784	putative protein kinase [Oryza sativa (japonica cultivar-group)]	1.2	7.0	0.00509
3996268		1.4	6.1	0.00970
3996734	nucleoid DNA-binding-like protein [Oryza sativa (japonica cultivar-group)] >gi|22775625|dbj|BAC15479.1|	1.1	10.2	0.00053
3996963	putative RNA polymerase III [Oryza sativa (japonica cultivar-group)] >gi|29788820|gb|AAP03366.1|	-5.3	-6.8	0.00578
3998263		1.6	14.3	0.00006
3998486	unknown protein [Oryza sativa (japonica cultivar-group)] >gi|20303596|gb|AAM19023.1|	1.4	7.8	0.00273

Table 2. Continued.

gene ID	Description of related protein	FC	tValue	FDR
3999064	hypothetical protein [Oryza sativa (japonica cultivar-group)]	1.5	8.9	0.00118
3999866	putative myosin heavy chain [Oryza sativa (japonica cultivar-group)] >gi|46805521|dbj|BAD16972.1|	1.6	8.7	0.00131
4001389	putative protein kinase AFC1 [Oryza sativa (japonica cultivar-group)] >gi|15623815|dbj|BAB67874.1|	1.0	6.2	0.00921
4001669		-1.1	-6.2	0.00921
4002443	putative 4-alpha-glucanotransferase [Oryza sativa (japonica cultivar-group)] >gi|22093785|dbj|BAC07076.1|	1.0	12.9	0.00013
4002475	lipoxygenase [Zea mays]	-1.0	-6.5	0.00710
4002778	OSJNBa0042P21.26 [Oryza sativa (japonica cultivar-group)] >gi|20161752|dbj|BAB90668.1| B1156H12.1	1.1	8.4	0.00168
4003205	putative RNA methyltransferases [Oryza sativa] >gi|13174243|gb|AAK14417.1|	1.2	6.6	0.00690
4003259	disease resistance response protein-like protein [Oryza sativa (japonica cultivar-group)] >gi|34394264|dbj|BAC84745.1|	5.8	7.4	0.00348
4004043		-1.1	-6.5	0.00718
4005126		-2.1	-7.4	0.00365
4005161	unknown [Zea mays]	-1.3	-14.9	0.00005
4007383		-1.3	-9.5	0.00082
4007654	plastocyanin precursor [Hordeum vulgare] >gi|431920|emb|CAA82201.1| plastocyanin [Hordeum vulgare subsp. vulgare] >gi|481190|pir||S38255	-1.2	-8.9	0.00119
4007856	putative gamma-thionin [Castanea sativa]	1.2	8.1	0.00204
4008085	unknown protein [Oryza sativa (japonica cultivar-group)] >gi|41053140|dbj|BAD08083.1|	1.2	7.7	0.00281
4008146		1.4	7.2	0.00428
4008577	putative 3(2),5-bisphosphate nucleotidase [Oryza sativa (japonica cultivar-group)] >gi|34394007|dbj|BAC84031.1|	1.2	8.3	0.00180
4008787	unknown protein [Oryza sativa (japonica cultivar-group)] >gi|49387593|dbj|BAD25768.1|	-1.2	-7.4	0.00352
4008964	Indole-3-acetate beta-glucosyltransferase (IAA-GLU synthetase) ((Uridine 5'-diphosphate-glucose:indol-3-ylacetyl)-beta-D-glucosyl transferase) >gi|626043|pir||A54739	1.1	8.6	0.00144
4009913	ALPHA-AMYLASE INHIBITOR 5 (SI ALPHA-5) >gi|322864|pir||S28202 alpha-amylase inhibitor SIalpha5 - sorghum >gi|264605|gb|AAB25194.1|	11.3	12.2	0.00016

Table 2. Continued.

gene ID	Description of related protein	FC	tValue	FDR
4010041	elongation factor 1 beta' [Oryza sativa (japonica cultivar-group)] >gi|322851|pir||S29224 translation elongation factor eEF-1 beta' chain - rice >gi|232031|sp|P29545|	-2.4	-9.2	0.00101
4010100	B1065G12.16 [Oryza sativa (japonica cultivar-group)] >gi|20161614|dbj|BAB90534.1|	1.3	8.9	0.00118
4011023		-1.2	-6.8	0.00575
4012125		-1.3	-10.9	0.00033
4014007	P0516D04.27 [Oryza sativa (japonica cultivar-group)] >gi|20804716|dbj|BAB92403.1|	-1.2	-6.6	0.00665
4014757		-1.7	-14.1	0.00006
4016306		1.5	10.2	0.00053
4016631	putative HSF-type DNA-binding protein [Oryza sativa (japonica cultivar-group)]	1.2	9.9	0.00065

*Fold change

Table 3. RT-PCR analysis for high and low starch samples for 15 and 20 DAP for the three populations assayed.

Population	Sample	DAP	FC[$]	Mean Ct Target	Mean Ct Reference	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
$(ILP^{90}\times B73)B73S_1$	HS[#]	15	1.64	33.91	32.24	1.68	0.37	0.77
	LS*			34.08	32.77	1.30		
	HS	20	12.94	32.48	30.26	2.23	-4.13	17.44
	LS			34.22	29.31	6.35		
$(ILP^{90}\times B73)B73S_3$	HS	15	NA[&]	33.29	32.60	0.70	-1.63	3.10
	LS			33.98	31.65	2.33		
	HS	20	NA	27.84	26.50	1.34	-5.83	56.81
	LS			33.44	26.27	7.17		
$[(ILP^{90}\times B73)B73S_3]$Fr616	HS	15	15.62	30.53	31.59	-1.07	-3.33	10.05
	LS			34.06	31.80	2.26		
	HS	20	15.03	33.76	31.77	1.99	-0.37	1.29
	LS			34.50	32.14	2.36		

[#] High starch families
*Low starch families
[$] Fold change measured in microarrays
[&] Not applicable

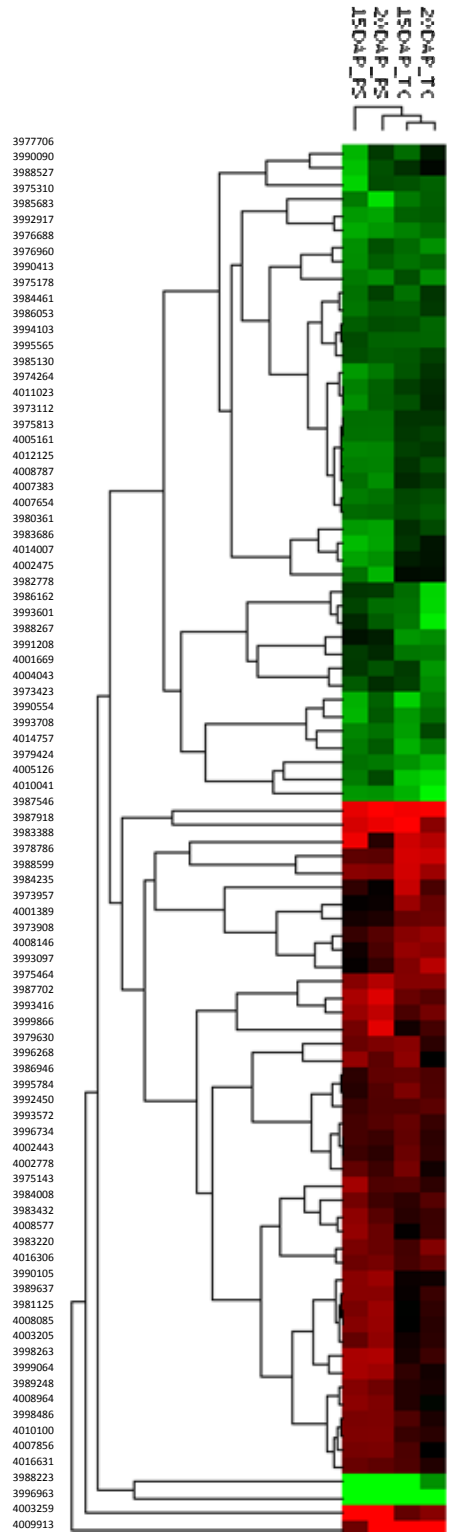

Figure 1. Clustering of selected genes and arrays for the $(ILP^{90}\times B73)B73S_1$ and $[(ILP^{90}\times B73)B73S_3]$Fr616 populations. From left to right: Gene ID, gene cluster, and heatmap. In heatmap red indicates the gene is up regulated, green indicates the gene is down-regulated, and black indicates a value close to zero. Cluster of the arrays is on top of heatmap where PS stands for $(ILP^{90}\times B73)B73S_1$ and TC for $[(ILP^{90}\times B73)B73S_3]$Fr616 populations, respectively.