Maize selection passes the century mark: a unique resource for 21st century genomics

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The Illinois Long-Term Selection Experiment for grain protein and oil concentration in maize (*Zea mays*) is the longest continuous genetics experiment in higher plants. A total of 103 cycles of selection have produced nine related populations that exhibit phenotypic extremes for grain composition and a host of correlated traits. The use of functional genomics tools in this unique genetic resource provides exciting opportunities not only to discover the genes that contribute to phenotypic differences but also to investigate issues such as the response of plant genomes to artificial selection, the genetic architecture of quantitative traits and the source of continued genetic variation within domesticated crop genomes.

Maize (*Zea mays*) has perhaps the longest recorded history as a model system for plant genetics, with many investigations being conducted before the rediscovery of Mendel's laws in 1900 [1]. Only one of these studies survives for exploration in the genomics era: the Illinois Long-Term Selection experiment (Figure 1). Beginning in 1896, 163 ears from the open-pollinated variety Burr's White were analysed for oil and protein concentration, for the purpose

of testing whether breeding and selection could improve kernel composition for feed and processing traits [2]. The 24 highest-scoring ears for protein formed the ILLINOIS HIGH PROTEIN STRAIN (IHP) (see Glossary), the 24 highest ears for oil formed the ILLINOIS HIGH OIL STRAIN (IHO) and the lowest 12 ears for these traits became the parents for the ILLINOIS LOW PROTEIN STRAIN (ILP) and the ILLINOIS LOW OIL STRAIN (ILO). Recurrent selection in these four populations has been performed every year since, except for three years during World War II, making the Illinois Selection Strains the longest running plant genetics experiment in the world.

The most striking observation in the Illinois Long-Term Selection Experiment is the significant degree of genetic variability remaining in these populations after more than 100 CYCLES of selection. Selection response continues in all the populations except ILO and ILP, which have probably reached lower biological limits for these traits owing to their poor germination frequencies and lack of change in recent cycles [3]. Further evidence for significant genetic variability is provided by the reverse selection experiments initiated at cycle 48 [4]. Trait values were reversed in each of the reverse selection strains at rates per cycle
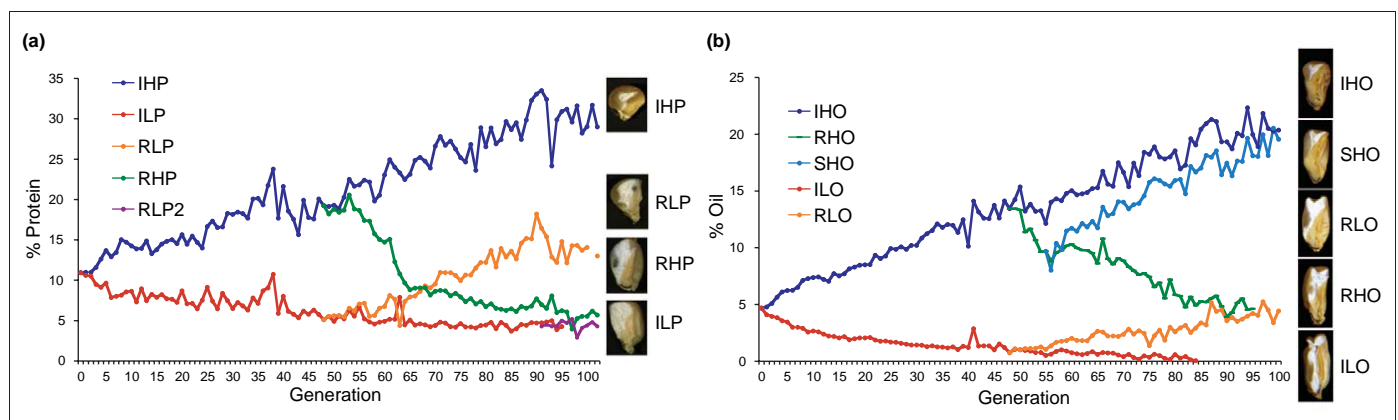
Figure 1. Selection responses in the Illinois Protein Strains (**a**) and Illinois Oil Strains (**b**). Selection has been performed for 103 cycles in each of the Illinois High Protein (IHP), Illinois Low Protein (ILP), Illinois High Oil (IHO) and Illinois Low Oil (ILO) strains. Selection was reversed in each of these four strains beginning at cycle 48 to produce the Reverse High Protein (RHP), Reverse Low Protein (RLP), Reverse High Oil (RHO) and Reverse Low Oil (RLO) strains. The Switchback High Oil (SHO) strain was initiated from RLO at cycle 55 and Reverse Low Protein 2 (RLP2) was initiated from ILP at cycle 90. Each cycle measured grain from 60–120 plants, with seeds from the highest or lowest 20% (depending on the direction of selection) selected to form the next generation. Grain was produced by controlled pollinations among sibling plants to minimize inbreeding. Cross-sections of mature kernels from cycle 100 of nine strains (all except RLP2) show phenotypic differences in protein (largely localized in tan areas at the periphery of kernels), starch (white areas), seed size and scutellum size (yellow tissue at right in each kernel). The selection response graphs are adapted from Ref. [3].

Glossary

Cycles: in selection experiments, a cycle is one round of genetic recombination, measurement of trait and selection of individuals for the next generation. In the Illinois Selection Experiment, a cycle is equivalent to a single generation; however, in other selection experiments involving different breeding or trait evaluation schemes (such as testcrosses and evaluation of hybrids), a single cycle might involve multiple generations.

Dosage effects: quantitative effects of gene action that are proportional to the number of alleles present. For example, in the triploid endosperm tissue, two doses of an allele come from the maternal parent and one from the paternal parent. If the alleles from the two parents show quantitative differences in phenotypic expression, they will exhibit dosage-dependent phenotypic effects when transmitted through either the maternal or paternal parent.

Epigenetic: heritable phenotypic differences that are not caused by changes in DNA sequence, but instead modifying factors such as the environment.

Illinois High Oil strain (IHO): population resulting from 103 cycles of selection for grain with high oil concentration.

Illinois Low Oil strain (ILO): population resulting from 103 cycles of selection for grain with low oil concentration.

Illinois High Protein strain (IHP): population resulting from 103 cycles of selection for grain with high protein concentration.

Illinois Low Protein strain (ILP): population resulting from 103 cycles of selection for grain with low protein concentration.

Imprinting: the selective expression of alleles at a given locus depending on which parent they were inherited from. As an example, some alleles of zein genes are only expressed when inherited from the maternal parent [38].

Linkage disequilibrium: the nonrandom association of alleles at different loci at frequencies that deviate from the expected random combinations of their frequencies in the population.

Near-isogenic lines: genotypes that are identical except at one or a few gene loci.

Overdominance: occurs when the phenotype of a heterozygous (Aa) genotype is superior to, or is outside the range of, phenotypes conditioned by the two homozygous (AA or aa) genotypes.

Restriction fragment length polymorphism (RFLP): difference in size (e.g. polymorphism) of a DNA fragment detected by a given DNA hybridization probe in a DNA gel blot analysis, indicative of a DNA sequence difference between alleles.

Reverse High Oil strain: population resulting from 48 cycles of selection for grain with high oil concentration, followed by 55 cycles for grain with low oil concentration.

Reverse High Protein strain: population resulting from 48 cycles of selection for grain with high protein concentration, followed by 55 cycles for grain with low protein concentration.

Reverse Low Oil strain: population resulting from 48 cycles of selection for grain with low oil concentration, followed by 55 cycles for grain with high oil concentration.

Reverse Low Protein: population resulting from 48 cycles of selection for grain with low protein concentration, followed by 55 cycles for grain with high protein concentration.

Switchback High Oil strain (SHO): population resulting from 48 cycles of selection for grain with high oil concentration, followed by seven cycles for low oil concentration, then 48 cycles for high oil concentration.

Targeting-induced local lesions in genomes (TILLING): a method that uses the polymerase chain reaction (PCR) to identify point mutations in a gene of interest by screening pools of chemically mutagenized lines [20].

similar to or greater than forward selection, with the SWITCHBACK HIGH OIL STRAIN (SHO) demonstrating that selection progress could again be reversed. What is even more remarkable about these selection responses is that, by cycle 17, all the selected individuals in each of the four strains trace back to a single ear (different for each strain) of Burr's White [5].

The Illinois Selection Strains have been intensively studied from the perspectives of quantitative genetics applied to plant breeding [3] and the physiological basis for differences in kernel composition [6]. Several other important concepts in maize breeding were first practiced in or developed from this classic plant genetics experiment (Table 1). However, little is known about the molecular basis for the observed phenotypic responses. The purpose of this article is to highlight the key features of the Illinois

Selection Strains that emphasize the value of performing long-term selection experiments as resources for plant functional genomics and studies of plant genome evolution.

Phenotypic responses to divergent selection for kernel composition

The Illinois Selection Strains span the known phenotypic extremes for maize kernel composition (normally 8–12% protein and 4–6% oil), which demonstrates the power of long-term selection in altering the expression of complex traits. Selection responses of both protein and oil are greater than 20 standard deviations from the original population mean in the positive direction and four standard deviations in the negative direction [3]. For quantitative traits such as kernel composition, these extreme phenotypic changes are not often possible through mutagenesis or transgenic approaches. Thus, long-term selection experiments not only have value for basic research on plant traits and their evolution but also have a practical use in crop improvement [7].

Selection experiments have the advantage of affecting many regions of the genome simultaneously, which can reveal relationships among interacting genetic pathways. The Illinois Selection Experiment has significantly altered multiple gene expression networks connecting seed development and physiology with processes operating throughout the entire maize plant (Figure 1, Table 2). Some of these responses in correlated traits were evident even by cycle 10 of the experiment [2]. Kernel starch concentration, and hence grain yield, is negatively correlated with both protein and oil concentration, presumably because starch production is energetically more efficient than protein deposition or oil synthesis. Selection for oil has also modified the relative proportions of embryo, specifically the oil-rich scutellar tissue, and endosperm in the oil strains (Figure 1). Thus, the Illinois Oil Strains offer opportunities to investigate the partitioning of carbohydrates between different tissues within the kernel. Protein is the storage form for nitrogen in the seed, and so divergent selection for grain protein concentration has also dramatically changed whole-plant nitrogen metabolism [6]. The Illinois Protein Strains therefore represent a novel genetic system to study source−sink relationships and nitrogen use efficiency in maize. Other phenotypes that differ among the Illinois Selection Strains (Table 2) might be associated with seed composition, but could also reflect the effects of genetic drift, selection for traits that facilitated the breeding scheme used or the hitchhiking of variant alleles owing to linkage with important factors for protein or oil concentration.

Illinois Long-Term Selection Experiment has affected many genes

The progressive phenotypic responses for kernel composition and correlated traits observed in the Illinois Selection Strains provide convincing evidence that these traits are controlled by many genes. A key question then becomes: how many genes are involved and can they be identified? Initial attempts to answer this question used the methods of quantitative genetics. Crosses between the

Table 1. Important historical contributions of the Illinois Long-Term Selection Experiment to plant breeding and genetics

Year	Contribution	Refs
1896	First planned experiment to improve cereal grain quality through breeding	
1897	Invention of the ear-to-row method of breeding and first use of the progeny test in maize	[41]
1910	Source of earliest inbred strains specifically developed to make hybrids. Two inbred lines developed from cycle 11 were parents in the first commercial double-cross maize hybrid grown in 1917	[42]
1949	Illinois High Oil was the major germ-plasm source for the first high-oil maize hybrids	[43]
1962	First demonstration of significant genetic variability in selected plant populations	[3]
1967	First use of non-destructive nuclear magnetic resonance to estimate seed concentrations of starch, protein and oil	[44]
1974	First use of near-infrared reflectance to estimate seed concentrations of starch, protein and oil	[45]
1975	First empirical evidence for linkage disequilibrium in breeding populations and its dissipation by random mating	[15,16]

different Illinois Selection Strains [8] showed that kernel protein and oil concentrations are largely determined by additive effects, with heterosis playing only a minor role. Estimates of the effective number of genetic factors controlling these traits range from 102 to 178 for protein and 14 to 69 for oil [3,9]. As expected from the presumed fixation of some alleles after 48 cycles of forward selection, comparisons of the reverse strains to their parental population estimated fewer effective genetic factors (10–56), with protein again having more than oil [3].

Mapping experiments have associated chromosomal segments with the phenotypic variation observed in populations derived from the crosses IHP × ILP [10–13] or IHO × ILO [14]. Several significant quantitative trait loci (QTLs) were identified for kernel starch, protein and oil concentrations, as well as other traits such as kernel weight, flowering date and plant height. Fewer QTLs were found for oil concentration than for starch and protein concentration, which is consistent with the prior estimates for the effective number of genetic factors. Many QTLs were significant for both starch and protein concentration, and hence might contain important factors acting on these inversely correlated traits. Significant QTLs were also detected for broad chromosomal regions containing candidate genes known to affect kernel composition [10], such as the *shrunken2* locus, which encodes the large subunit of

ADP–glucose pyrophosphorylase, the rate-limiting enzyme for endosperm starch synthesis.

Clusters of linked markers were significantly associated with some QTLs in the above mapping experiments, suggesting the presence of multiple closely linked genes contributing to a trait within a chromosomal segment. These results are expected because significant LINKAGE DISEQUILIBRIUM (LD) was demonstrated for crosses among the Illinois Selection Strains [15,16] that was greatly reduced following four generations of random mating. More recently, molecular marker analysis of the randomly mated IHP × ILP population [13] verified that random mating greatly reduces LD. This reduced LD leads to improved precision of marker–QTL associations, but fewer significant QTLs are detected. The number of randomly mated generations has been extended to eight for the IHP × ILP cross and to ten for IHO × ILO. In the randomly mated IHO × ILO population, ~50 QTLs for oil have been identified (Cathy Laurie and John LeDeaux, pers. commun.), suggesting that improved mapping resolution can also increase the number of significant QTLs. These populations will be of tremendous value for gene discovery through the positional cloning of the genes that are responsible for observed QTLs. In addition, they can potentially break tight linkages between genes that have opposing effects on kernel composition traits, which

Table 2. Correlated traits altered in the Illinois oil and protein selection strains

Trait	Correlation with grain protein concentration[a]	Correlation with grain oil concentration[a]	Refs
Grain starch concentration	−	−	[3]
Seed size	−	−	[3]
Proportion of embryo scutellar tissue	No correlation	+	[3]
Endosperm storage protein synthesis, particularly α-zeins	+	No correlation	[17,35,46]
Nitrogen assimilation, translocation and remobilisation	+	No correlation	[37,47,48]
Activity of nitrogen and carbon metabolism enzymes	+	No correlation	[35,49–51]
Leaf senescence or 'staygreen'	−	No correlation	[6]
Seed phytate content	+	−	[52]
Endoreduplication in leaf epidermis and endosperm	+	No correlation	[53,54]
Duration of juvenile vegetative phase	Reduced in Illinois Low Protein	No correlation	[b]
Relative maturity	Delayed in Illinois High Protein, Reverse High Protein, REVERSE LOW PROTEIN	Delayed in Illinois Low Oil and Reverse Low Oil	[3]
Tassel branching	No correlation	High in Illinois High Oil	[55]
Resistance to foliar fungal pathogens and insect pests	Poor in Illinois High Protein and Reverse High Protein	Poor in Illinois High Oil and Illinois Low Oil	[c]

[a] +, positive correlation; −, negative correlation.
[b] S. Moose, unpublished.
[c] J. Dudley and S. Moose, unpublished.

might be responsible for the inverse relationships between starch and protein or starch and oil concentrations.

Illinois Selection Strains as a functional genomics resource

The Illinois Selection Strains represent the known genetic extremes for seed composition and a suite of correlated traits that contribute to the agronomic performance and economic value of maize as a crop yet are poorly understood at the molecular level. These dramatic phenotypic differences, coupled with the availability of well-developed genomics resources for maize, make the Illinois Selection Strains a unique, valuable and untapped resource for elucidating gene function in cereal crops. Because many genes are predicted to be involved and the vast majority of them are unknown, functional genomics approaches are warranted.

The accumulation of the zein endosperm storage proteins and mRNA expression of the major α-zeins differs dramatically among the Illinois Protein Strains [17], as would be expected for populations showing a nearly eightfold difference in grain protein concentration. However, gene expression analyses with the Illinois Selection Strains have been complicated by the intentional maintenance of these strains as genetically heterogeneous populations. Recently, inbred lines have been generated from cycle 90 of the Illinois Protein Strains that exhibit phenotypes representative of the cycle 90 populations [18]; these will greatly facilitate comparisons using global mRNA, protein and metabolite profiling. Preliminary characterizations of these Illinois Protein Strain inbred lines have found differences in the expression of key regulatory genes for zein synthesis as well as the amounts and profiles of amino acids during vegetative growth and the grain-filling period (S. Moose and F. Below, unpublished).

The challenge of mRNA expression profiling or proteomics experiments often lies in the functional validation of the many candidate genes identified. The Illinois Selection Strains and materials derived from them offer an array of genetic tools and resources that will facilitate the functional validation of candidate genes in a parallel manner. The reverse selection strains provide replicate tests in closely related genetic backgrounds of gene expression changes that can be associated with a phenotype. For example, a gene whose expression is positively correlated with high oil concentration would be expected to be more highly expressed in IHO, SHO and the REVERSE LOW OIL STRAIN compared with ILO and the REVERSE HIGH OIL STRAIN. Furthermore, such genes should not show significant expression differences among the four protein strains that do not vary in oil concentration. The inbred lines derived from the Illinois Selection Strains can be used to test for gene expression changes in response to defined physiological treatments. To facilitate such studies, mutations in genes regulating key steps in zein and starch synthesis, such as *opaque2* [19] and *shrunken2*, have been introgressed into the Illinois Protein Strain inbred lines to create NEAR-ISOGENIC LINES with defined alterations in endosperm storage pathways. Populations of the Illinois Protein Strain inbred lines have also been generated in

which mutations were induced using ethane methyl sulfonate. These mutagenized populations can be screened for novel mutant phenotypes in nitrogen metabolism and could be used as a future resource for TARGETING-INDUCED LOCAL LESIONS IN GENOMES (TILLING) [20] to screen for mutations in candidate genes.

The availability of selfed lines from the extensively randomly mated IHP × ILP and IHO × ILO populations provides powerful, high-resolution mapping resources to test whether candidate genes localize with QTLs for seed composition and correlated traits. These unique mapping populations might also be useful for combining mRNA and protein profiling with quantitative trait mapping, as demonstrated in two recent studies with maize [21,22]. When combined with the improving linkage and physical mapping tools for maize and rapidly expanding maize genome sequence information, quantitative trait mapping will soon become a viable gene discovery tool in maize.

The Illinois Selection Strains also offer unique opportunities to integrate linkage and LD mapping strategies, owing to the availability of more than 100 generations of recombination in populations of known population size and genetic structure. Similar combined approaches have been successfully used in animal genetics [23,24]. Selection is expected to alter allele and haplotype frequencies, and to increase local LD for genes that contribute to phenotypic differences. Thus, documenting that a particular chromosomal region shows evidence of selection relative to neutral sequences is one method to validate candidate genes contained within the region or to identify additional genes missed by profiling approaches. Such a strategy has been previously used to identify QTLs in divergently selected mouse (*Mus musculus*) and *Drosophila melanogaster* populations [25,26]. Similarly, Yves Vigouroux [27] used changes in allele frequencies between maize (*Zea mays* ssp. *parviglumis*) and teosinte (*Zea mays* ssp. *mexicana*) populations to discover genes showing evidence of selection during maize domestication that might be associated with agronomic performance. The extended LD in the Illinois Selection Strains relative to diverse maize populations can decrease the resolution potentially available with association mapping [28], but could also be advantageous by requiring fewer markers for genome scans of chromosomal regions showing LD. Promising candidate genes and regions could then be investigated further using the higher resolution mapping populations generated by random mating of IHP × ILP and IHO × ILO crosses.

Eternal genetic variability within the Illinois Selection Strains?

The continued phenotypic responses after more than 100 selection cycles indicate that significant genetic variability remains in the Illinois Selection Strains. Two studies have examined genetic variation in these populations. Jay Sughroue and Torbert Rocheford [29] surveyed variability in 52 individuals from cycle 90 of the Illinois Oil Strains at 49 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) loci shown to be polymorphic between IHO and ILO. They found multiple alleles (mean of 2.73) at most loci (59%) whose frequencies varied significantly among the four

different strains. Of the RFLP loci in this study, 14 (20%) showed evidence of selection based on patterns of variation between the forward and reverse selection strains. More recently, Venu Mikkilineni and Torbert Rocheford [30] surveyed 25 individuals from cycles 65 and 91 of the Illinois Protein Strains for variation at 32 RFLP loci previously determined to be polymorphic between IHP and ILP. They found evidence for progressive inbreeding, yet higher than predicted diversity and heterozygosity in each of the strains. As for the Illinois Oil Strains, most markers showed differences in variant frequencies between the Illinois Protein Strains and selection cycles, some of which corresponded to markers previously associated with QTLs for grain composition. Both of the RFLP surveys are somewhat biased in that only markers demonstrated to be polymorphic between the populations were assayed; however, most of the RFLP probes screened detected polymorphisms, indicating significant genetic variability. Additional genome-scale scans of the Illinois Selection Strains using microsatellite markers or single-nucleotide polymorphisms will provide a more-detailed picture of the genetic variability present in these populations.

Two main models have been proposed to explain the source of continued variability in long-term selection experiments: (i) there are multiple alleles in the base population at many loci that have small effects on the phenotype (Fisher's infinitesimal model) [31]; and (ii) mutation generates new alleles that contribute to response after variation in the base population is exhausted [32]. Existing evidence favors the infinitesimal model for the Illinois Selection Strains, with mutation probably also contributing.

The infinitesimal model is supported by the many loci controlling protein and oil composition that are predicted from quantitative genetic estimates and observed in QTL mapping experiments. The degrees of allelic diversity and heterozygosity remaining in the Illinois Selection Strains are too high to be explained by mutation and are similar to surveys of genetic diversity among extant individuals from other open-pollinated maize varieties grown during the same era as Burr's White [33]. Furthermore, few RFLP variants are unique to only one of the four original populations or their reverse selection derivatives [29,30]. Each of these observations is consistent with the major prediction of the infinitesimal model: that variability is maintained because selection does not dramatically alter individual allele frequencies. The main argument against the infinitesimal model is that the effective population sizes for the Illinois Selection Strains are estimated to be only 4–12 individuals [34]. Less than 2% of the initial heterozygosity would still be expected to be present after 90 cycles of selection from an initial effective population size of 12, which is considerably less heterozygosity than observed [29,30]. Because each of the 300–500 kernels on a single ear could have different pollen parents, the effective population size is raised to a maximum of 96, which would lead to higher levels of heterozygosity. Other important factors that can contribute to the maintenance of genetic variation include balancing selection (in which OVER-DOMINANCE retains heterozygosity) and the fact that alleles at some loci might initially be neutral but then become advantageous or deleterious in later selection cycles owing to changes in genetic background mediated by loci with large effects. Such mechanisms might explain the phenotypic responses observed in the reverse selection experiments.

A potential role for mutation in the Illinois Selection Strains is provided by two observations. The first is the rapid rate of response during the first 17 selection cycles in the REVERSE HIGH PROTEIN STRAIN (Figure 1), which could be explained by the fixation of mutations with large effects that might have occurred during the previous 48 cycles of forward selection in IHP. The second is the frequent appearance of recessive mutant phenotypes (e.g. chlorophyll-deficient seedlings, defective kernels and reduced plant height) upon inbreeding any of the nine Illinois selection strains (S. Moose and J. Dudley, unpublished). It is important to emphasize here that a range of processes could be operating to generate new allelic variants collectively grouped as mutations, including transposable-element activity, single-base changes, small insertions or deletions, or even intragenic recombination in which haplotypes from two existing alleles are combined to produce a novel allele with a significantly different effect on a trait.

Finally, although not strictly representing mutation, EPIGENETIC variation in gene activity could also be contributing to phenotypic differences. One potential example of epigenetic control of gene expression in the Illinois Protein Strains is the strong maternal inheritance of grain protein concentration in reciprocal crosses of IHP and ILP [35,36]. These phenotypes cannot be fully explained by the influence of the vegetative source on kernel sink traits [37], suggesting that parental IMPRINTING and DOSAGE EFFECTS within the triploid endosperm might be affecting zein gene expression in IHP and ILP, as has been observed in other maize genotypes [38,39].

Prospects for future work with long-term selection experiments in plants

Many other long-term selection experiments (exceeding ten cycles) have been conducted in maize for a range of traits, most selecting for improved grain yield (Table 3). In addition, modern commercial elite maize inbred strains could be viewed as the product of a large, long-term selection experiment for grain yield [40]. Unlike counterpart selection experiments in mammals or *D. melanogaster*, there are seed samples of different selection cycles that can be readily grown and reproduced for new experimentation. For example, selection responses might be assessed in different environments to estimate genotype-by-environment interactions, such as growing the Illinois Protein Strains under different rates of supplementary nitrogen, a factor known to influence grain protein concentration [18]. Long-term selection experiments are likely to be successful in any plant species, including those that are primarily self-pollinated (such as *Arabidopsis*), because dramatic selection responses have been observed in many experiments using inbred lines [32]. The Illinois Selection Strains demonstrate the tremendous power that these experiments have as resources for discovering genes controlling quantitative

Table 3. Other maize long-term selection experiments (more than ten cycles) potentially available for study

Trait(s)	Duration (cycles)	Refs
Increased grain oil concentration	28	[43]
Divergent selection for ear length	27	[56]
Prolificacy (ears per plant)	24	[57]
Aluminum tolerance	22	[58]
Stalk crushing strength	16	a
Rind penetrometer resistance and stalk strength	15	[59]
Nitrogen use efficiency, prolificacy, grain yield	14	[60]
Grain yield	10–25	[61]

[a]L. Darrah, unpublished.

traits and for investigating the dynamics of evolutionary change in plant genomes. The application of genomics tools to long-term selection experiments promises to reveal insights into plant evolution that will have impacts throughout the 21st century and beyond.

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