INTERROGATING THE GENETIC VARIATION OF INBRED PLANTS

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Plants are at the mercy of environmental variation. Without locomotion or behavior, they must rely on genetic variation to cope with changes in the environment. How is this accomplished by inbreeding plants? More than half of all annual plants are inbreeding, including most important agronomic plants such as soybean (*Glycine max*). As a consequence they are homozygous and maintain their functional genomes intact during reproduction. Genetic variants that arise will rapidly become homozygous and such variation will be maintained in successive generations. How do inbreeding plants evolve adaptation to their environments? What is the source and nature of their genetic variation?

Here we discuss three aspects of genetic research that suggest answers to these questions: 1. Changes in amino acid repeats within genes can arise with high frequency due to the expansion or contraction of nucleotide repeat sequences. Such variation would allow rapid, focused and reversible response of plants to environmental change. 2. Mutations occurring in somatic cells can give rise to seed, providing a large cellular target for mutational change. 3. The functional genome of inbreeding plants is not disrupted during reproduction. Therefore, within the genome interactions can adapt in the phenotype to environmental change. This interaction between genetic variants presents the entire genome as a target for genotypic variation. Thus, the plant genome itself is also an extremely large target for genetic variation.

Homage to the past

In 2002, I began collaborating with Dr. Lyudmila Trut at the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. Through that work, I became acquainted with the research and career of Dimitri Belaeyev. As my knowledge of his career grew, so did my respect for his insights and intuition. It is my great loss that I never met him nor had the opportunity to sit down and discuss evolutionary questions with him.

The question that I address here is one that I am sure would have interested him greatly. The simplicity of the question is typical of all great problems: How does an inbreeding organism derive and maintain the genetic variation that allows it to cope with its environment? We accept this genetic variation without recognizing that it is remarkable, a puzzle demanding a solution!! It is exactly such mysteries that challenged Belaeyev and recognizing them, as such, placed him in the first rank of great scientists.

The question

Unlike animals, plants lack locomotion and/or behavior to avoid the vicissitudes of changing environments. More than 50 % of all plants are inbreeding as are ca. 70 % of crop plants (Stebbins, 1950; Allard *et al*., 1968; Allard, 1975; Glémin, 2006). Over centuries, man has adapted such plants to his own purposes carefully selecting for properties such as yield, seed weight, lack of shattering, absence of lodging, adaptation to climates that vary in photoperiod, rainfall and soil composition. This adaptation process continues today as plant breeders improve yield and adapt inbred plants to different environments. *How does an inbred plant acquire its genetic diversity*? Inbred plants do not outcross; they are homozygous inheriting their entire genome unchanged except for those mutational changes in the genome that occur within the plant itself. Diversity within or between populations cannot serve as a source of individual genetic variation.

In what follows, I shall argue that three parameters allow inbred plants to generate

the amount of genetic variation required to heritably adapt to changing environments and to selections imposed by man: 1) Mechanisms of mutation have been discovered that are much more frequent than single nucleotide change (These are intrinsically variable and most importantly, reversible, preventing fixation and thus maintaining the process of heritable adaptation to changing environments); 2) the target for mutational change within the plant is extremely large; and 3) combinatorial interactions between different portions of the genome vastly increases the ability to provide the variation required to adapt to the environment.

Intrinsic variation – repeated sequences

Tandemly repeated DNA sequences undergo slippage mutations with high frequency, are locus specific, have been shown to have incremental effects on phenotypic function, and can contract or expand producing reversible effects on the phenotype (Fondon, Garner, 2004; Kashi, King, 2006). Mutation rates «forward or backward», typically between 10^{-2} and 10^{-4}, prevent single alleles from remaining fixed and in the absence of selection variation will proceed randomly in both directions. Such mutations have been identified in a number of animal systems often with startling effects on the phenotype. Thus the effects of polyalanine repeats characterize the nuclear or cytoplasmic localization of transcription factors (Albrecht *et al*., 2004). Cis regulatory effects of sequence repeats on transcription have been reviewed by Rockman and Wray (2002), with the conclusion that such effects may have a profound effect on a wide variety of phenotypes.

Perhaps the most startling example and most relevant to plant adaptation is the per gene in *Drosophila*, which contains repeated sequence coding for a dipeptide of alternating threonine and glycine residues. This gene controls the circadian rhythm of behaviors (Hall, 2003; Rogers *et al*., 2004; Sawyer *et al*., 2006). In natural *Drosophila melanogaster* populations, cline dependent allele frequency occurs in a reproducible manner on multiple continents and different geographic regions suggesting that sequence slippage mutations in the Thr-Gly repeat within the *per* gene provide a rapid response to environmental change.

As yet, little is known about the occurrence, polymorphic frequency or effects on phenotype of repeat sequences in plants. However, emerging research on the sequence of multiple individuals from different subspecies (races) of *Arabidopsis*, located in different environments should shed light on the role that such mutations play in plant adaptation. As sequencing technologies become more economic and rapid, the effects of adaptation on repeat sequence variation in crop plants should also become apparent. It seems likely that contraction and expansion mutations of repeat sequences will play an important role in the adaptive phenotypic variation of inbred plants.

Other types of mutation such as transposon insertion/deletions or cassette exchanges (gene conversions) can play similar important roles although the phenotypic changes controlled by these will tend to be more absolute. Because inbreeding plants self-pollinate mutations rapidly become homozygous and expressed in subsequent generations, becoming subject to selection that can either increase or eliminate the variant population within a short time.

Soma vs germplasm

Unlike animals, plants do not sequester their germplasm. Individual somatic cells can differentiate to form reproductive organs and eventually seed, serving as a source of new germplasm. This simple fact implies that seed can arise from soma derived from cells and ramets that have been under selection during vegetative growth[1] As a result, large populations of vegetative cells can serve as the target of mutational events that can be

[1] Retrospectively it is interesting to note that much research carried out in Russia on plants during the period between World Wars 1 and 2, focused on the apparent heritability of changes selected during growth. This non-Mendelian behavior, Lamarkian in many aspects, profoundly influenced the course of genetics in Russia during subsequent years. We now understand that because the germplasm of plants is not sequestered, changes in somatic cells can enter the germline and, under certain selective conditions, vegetative growth of mutant cells will be favored and can produce altered ramets that ultimately will produce gametes and seed. In those instances, selection is acting within the plant on vegetative cells that can eventually give rise to germplasm. This difference between plants and animals has profound consequences on life histories of plants and their influence on subsequent progeny. It is unfortunate that such an interesting phenomenon has been largely ignored because of its historical use to further political agendas unrelated to science.

incorporated into gametes and subsequently into seed. This increase in target size will be of moderate size in annual plants, but can become quite large in perennials, in which the regulation of vegetative growth by apical meristems can selectively increase the number of mutant cells that are able to multiply under adverse conditions (Lark, 1985). Thus, like the seed bank, the ramets of a perennial plant become a library of selected genetic responses to environmental variation associated with the individual plant's life history. The reproductive process of gamete and seed formation then serves as a bottleneck that cleanses the mixture of vegetative genomes sequestering different genomes into individual plants. Although this process is the same for outcrossing and inbreeding plants, it is only in the inbred lines that genomes become homozygous within a generation and can be selectively increased or lost depending upon the environment. Moreover, the presence of homozygous, mutant, seed in the seed bank can test environmental variation in succeeding years.

Combinatorial variation: the genome as a target for change

Because the homozygous genome of inbreeding plants is not genetically disrupted during reproduction, interactions between genes in different regions of the genome will be inherited and subject to positive or negative selection. Whereas in outcrossing plants interactions can only persist when they involve closely linked genes, within inbreeding plants the entire genome can participate. Thus the target of combinatorial change is vastly greater. Moreover, the fact that the entire genome can experience mutational events that participate in interactions, again greatly increases the target for mutation.

Experiments from our laboratory, using Soybean, illustrate different aspects of interaction that regulate quantitative traits.

Three recombinant inbred (RI) populations were developed (MN, MA, NA) by crossing three parental inbred lines (Fig. 1) (Mansur *et al*., 1996). Each line was genotyped using molecular markers (Song *et al*., 2004). In each line, marker loci and Quantitative Trait Loci (QTLs) are homozygous derived from either of two parental genotypes (M, N, or A) (e.g. in the MA population every locus is either AA or MM etc.).

Various agronomic traits have been identified in each population and mapped to loci associated with markers. Computational techniques developed to identify interactions between different loci (Chase *et al*., 1997) then demonstrated that interactions regulated a variety of agronomic traits including, among others, yield, seed weight, seed number and reproductive period.

In order to study the nature of these interactions more extensively, a model trait was examined under conditions that allowed us to analyze the effects of genetic background, and environment on different aspects of the phenotype. Soybean is a dicotyledonous plant that grows with a branching structure with leaf bearing nodes. Normally, each such node gives rise to a trifoliolate leaf. However, it has been known for some time that certain genotypes can give rise to tetra and penta foliolate leaves (Fig. 2; see also Fehr, 1972). Moreover, it had been shown that this property was genetically determined and expressed with low penetrance. Two phenotypes could be shown to segregate: the frequency of plants bearing abnormal leaves and the number of abnormal leaf nodes per plant (hereafter referred to as plants per row and nodes per plant respectively). These phenotypes segregated in the RI populations.

The three RI populations were grown together in Minnesota USA, summer of 2001 and in addition, one population (MN) was grown in the

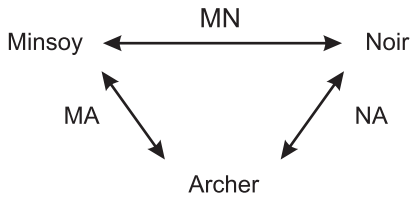

Fig. 1. Arrows indicate genetic crosses between parental accessions used to produce the three populations of recombinant inbred plants.

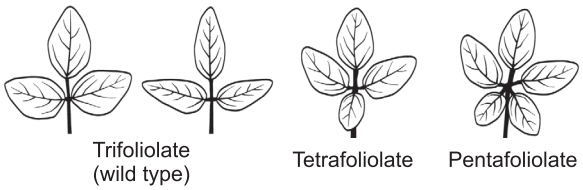

Fig. 2. Normal (trifoliolate) and mutant (tetrafoliolate) leaves.

same location during 2002 (For details see ref.). Thus, we could compare different genotypes in one environment and one genotype in two environments. Table 1 summarizes the mean frequencies and range of value observed for each genotype in each environment. The origin of the three populations (MN, MA, NA) is described in Figure 1. It can be seen that both the means and the range of values varied with the genotype and the environment.

Because we can identify QTLs and calculate the significance of their association with markers, we can compare the influence of specific QTLs for each phenotype when expressed in different genetic backgrounds and different environments. Figure 3 summarizes all of the loci that could be identified using the three RI populations in the two environments. Seventeen loci on 11 different chromosomes could be identified. Some loci affected both phenotypes, but others discriminated between plants per row and nodes per plant. In addition, we identified 11 interactions between loci (for details of these experiments see Orf *et al.*, 2006).

Table 1

Multi-foliolatre variation:
Genotypes and environments

RIL	Pheno/env	Mean	Range %
MN	PI/row ‘01	13.3	0–85
MN	Nd/PL ‘01	10.0	0–35
MN	PI/row ‘02	24.5	0–100
MN	Nd/PL ‘02	20.0	0–90
NA	PI/row ‘01	8.2	0–40
NA	Nd/PL ‘01	8.1	0–80
MA	PI/row ‘01	5.6	0–70
MA	Ns/PL ‘01	6.0	0–30

Multi-foliolate frequency: Pl/row: % of plants; Nd/PL: % nodes/plant.
Environments: Minnesota — ‘01 & ’02.

Table 2 presents examples of interactions of QTL haplotypes with their genotypic background (A, N, or M,), and with their environment. In example **a**, the background containing N and M expresses the genotype, but substituting A for either M or N in the genotypic background prevents expression. In example **b**, the substituting A for M alleles changes the phenotype affected from plants/row to nodes/plant. Finally, in example **c**, QTLs shown to be active in one environment are not active in another. These examples, taken from the QTLs in Figure 3, illustrate the interaction of specific loci with the rest of the genome or with the environment. For a more extensive review of these data see Orf *et al*. (2006).

Values were adjusted to a population mean of zero. Means of specific genotypic subpopulations varied around this mean (values were therefore either positive or negative). For each example, the phenotype (trait) is listed followed by the parent alleles corresponding to homozygous loci from the A, N, or M, parent populations. Alleles are paired to indicate AN, AM or MN RI populations. Significant variation is indicated in **bold**, very significant variation is **boxed**.

The sign of the mean value indicates whether the allele increases or (-) decreases the mean value of the genotypic subpopulation.

A. A QTL that regulates the frequency of both plants/row and nodes/plant is only active in the MN population. Variation is *not* regulated by this QTL in the MA or NA populations.

B. A QTL is only active in the MN and NA populations. It regulates the frequency of plants/row in the MN population, but the frequency of nodes/plant in the NA population.

C. Two QTLs regulate the frequency of plants/row in the MN population: U12-2 is active only in the 2001 environment, U14-2 is active only in the 2002 environment.

In Figure 3 we summarized specific interactions identified between unlinked loci (lines between boxes). Several are involved in multiple interactions (Table 3). Unfortunately, the size of the RI populations analyzed did not provide the analytical power to determine if interactions involved pairs of loci separately or the simultaneous interaction of three, four or five at a time.

The phenotypic means of the four genotypic subpopulations produced by particular genotypic interactions reveal patterns that are suggestive. Table 4 presents several examples of interactions analyzed in two environments.

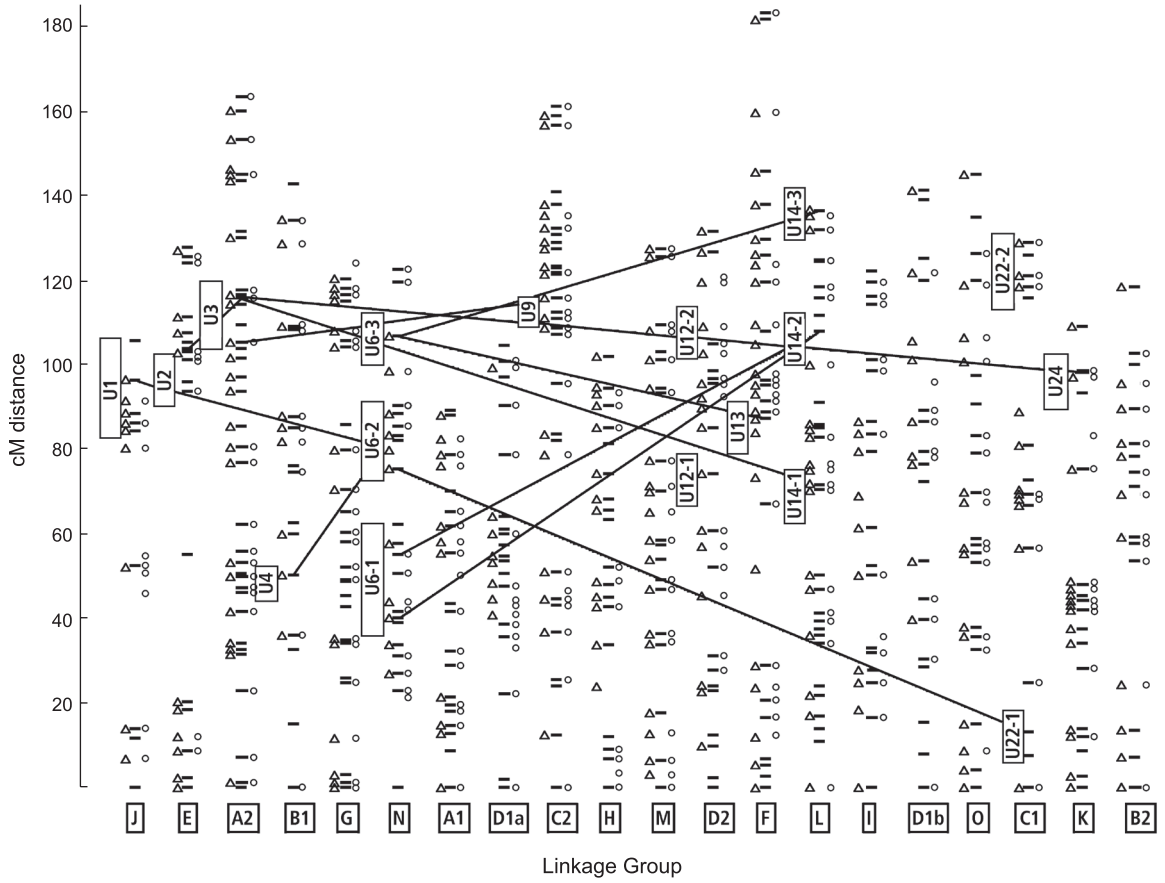

Fig. 3. Locations of QTLs regulating abnormal foliolate formation on the Soybean genetic map.
Δ – ○ are locations of SSR markers for the MA, MN, or NA RI populations respectively. QTLs are presented in boxes that define the limits of the region in which they are located. Lines between boxes denote interactions between QTLs.

Interactions may increase or decrease the frequency of multi-foliolate leaves. The interaction between the loci on U2 and U3 illustrates a particular pattern observed in both environments: one pair of genotypes, N_1N_2 increases the frequency of abnormal foliolate leaves, the other three genotypes do not exhibit any effect (their negative value is the result of adjusting the population mean to value «0»). This pattern is to be expected if the interaction between loci is allele specific, resulting from co-adaptation during evolution of the Noir genome.

Four genotypic populations are associated with each pairwise interaction. The limitation to four is the result of homozygosis at each of the four loci. Each locus (e.g. N_2, N_1, M_1M_2) is denoted by the parental allele (N or M) and the locus number (1 or 2) as a subscript. The genotype is presented as a haplotype that represents the homozygous diplotype.

As in Table 2, the mean of each genotypic population is given relative to an overall population mean of 0.

The interaction between U3 and U14-1 shows similar allele specificity, albeit less strong, in which interaction between Minsoy alleles (M_1M_2) reduces the frequency. The fact that M_1N_2 shows a slight tendency towards this phenotype suggests that it is the M_1 locus that plays an important role in determining the phenotype. That is to say, expression of the phenotype of the M_1 allele does not appear to be completely conditional upon its interaction with M_2.

A different pattern is displayed in by the interaction between U1 and U6-2 during the 2001 season. In this example, interactions between N_1 and either N_2 or M_2 do not appear to affect the phenotype, whereas interactions between M_1 and

Table 2

Examples of interactions with genetic background or environment

Linkage Group Map Region Nearest Marker		U6-1 40-62 Satt080	Linkage Group Map Region Nearest Marker		U14-3 130-138 A802_2	Linkage Group Map Region Nearest Marker		U12-2 102-110 L204_2	U14-2 100-112 G173_1
Trait	Parent allele		Environ-ment	Trait	Parent allele	Environ-ment	Trait	Parent allele	
	A	-0.01			A	-0.05			
	N	0.01			N	0.07			
Plants/ row (P/R)	A	0.07	MN 2001	Plants/ row (P/R)	A	0.02		N	0.05	**0.23**
	M	-0.06			M	0.05		M	-0.05	**-0.29**
	N	**-0.38**			N	**0.15**				
	M	**0.33**			M	**-0.21**				
	N	**-0.31**			N	0.09		N	**0.26**	0.03
	M	**0.35**			M	-0.12		M	**-0.23**	-0.04
Nodes/ plant (N/P)	A	-0.01	MN 2001	Nodes/ plant (N/P)	A	0.08	QTL effective in only one environment			
	M	0.01			M	-0.06				
	A	0.08			A	**-0.20**				
	N	-0.11			N	**0.26**				
a			**b**				**c**			

Table 3

Identification of pairwise interactions between QTLs affecting the frequency of multi-foliolate leaves

Locus 1	Locus 2
U3	U2
	U14-1
	U24
	U9
U6-2	U1
	U4
	U22
U6-3	U13
	U14-3

Notes: Three loci are listed on the left (Locus 1) together with the loci with which they interact on the right (Locus 2).

M_2 increase the frequency, interactions between M_1 and N_2 decrease the frequency. The pattern suggests an allele specific change in M_1 that promotes multi-foliolate leaves in the Minsoy parent in which it co-evolved with M_2; however, that same allele specificity makes interaction with N_2 no longer possible, decreasing the frequency of the phenotype. N_1 retains the ability to interact with either N_2 or M_2. A similar allele specific interaction is seen between U6-2 and U22, except that in this case the locus on U22 (N_2) has evolved in Noir in such a way that interaction with U6-2 (N_1) inhibits formation of multi-foliolate leaves, an inhibition that cannot occur when the Minsoy allele at U6-2 is paired with the Noir allele on U22 (M_1N_2).

Finally, interactions between U6-3 and U14-3 display different patterns during the 2001 and the 2002 seasons. The patterns suggest that the Noir

Table 4

Means of four genotypic subpopulations produced by interactions between pairs of QTLs that regulate the frequency of multi-foliolate leaves

Pairwise Genotypes		Minn '01		Minn '02	
		N_1N_2	N_1M_2	N_1N_2	N_1M_2
Locus 1	Locus 2	M_1N_2	M_1M_2	M_1N_2	M_1M_2
U2	U3	0.62	–0.29	0.59	–0.27
		–0.18	–0.21	–0.18	–0.19
U3	U14-1	0.26	0.21	0.19	0.25
		–0.09	–0.53	–0.05	–0.55
U1	U6-2	–0.18	–0.18	–0.14	–0.06
		–0.42	0.61	–0.31	0.40
U6-2	U22	–0.42	–0.11	–0.28	–0.08
		0.48	–0.09	0.44	–0.21
U6-1	U14-2	–0.26	–0.44	–0.37	–0.06
		0.61	–0.16	0.35	–0.03
U6-3	U14-3	–0.29	–0.20	–0.46	0.05
		0.53	–0.22	0.42	–0.09

locus on U14-3 co-evolved with the U6-3 Noir locus (N_1N_2) to inhibit multi-foliolate leaf production under the environmental conditions of the 2002 season. However, when paired with a Minsoy allele on U6-3 (M_1N_2) the frequency of unusual leaves is increased!! In the 2001 environment, the inhibited phenotype (N_1N_2) is not expressed, whereas the unusual enhancement (M_1N_2) is still seen.

These examples illustrate that one allele may interact specifically with one or more allele(s) at another locus, and that such interactions can be modified by interaction with the environment.

Modeling interactions

It is sometimes easier to visualize interactive effects if one imagines a phenotype produced by the interaction. This may be most important, when trying to understand how an evolutionary change can be deciphered from segregant progeny derived from distant parental lines. Minsoy and Noir are distant soybean cultivars (ref). In Figures 4 and 5, we present an hypothesis that illustrates how interactions between loci and the environment in segregant lines could influence a phenotype.

We assume that the coincidence of expression of the two loci brings about a change in the phenotype. The frequency of multi-foliolate plants or nodes depends on the length of this overlap period. Figure 4 suggests an evolutionary scenario in which Minsoy and Noir genotypes are originally identical and multi-foliolate leaf formation occurs when Locus 1 and locus 2 are being expressed concurrently during morphogenesis (Fig. 4A). The Noir locus 1 (N_1) undergoes mutation whereby its expression begins earlier than the loci N_2, M_1 or M_2 (Fig. 4B). A subsequent mutation in N_2 prolongs the expression of that locus (Fig. 4C). Neither of these changes affects the interaction (overlap period) of N_1 with N_2. Thus, although the genotype of Noir has changed, the phenotype remains the same.

The change in the genotype becomes evident when Minsoy and Noir are crossed and the RI segregant population is examined. Analysis of those segregants (Figures 5A and B) reveals a new phenotype corresponding to the genotype M_1N2

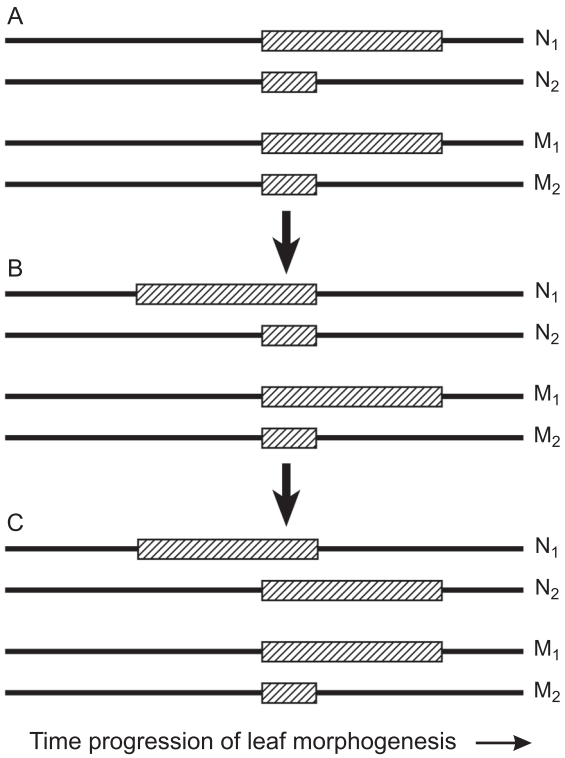

Fig. 4. Evolution of change between Noir and Minsoy. A – we imagine that locus 1 (M_1 or N_1) and locus 2 (M_2 or N_2) were identical for the two lines. B – N_1 changes in Noir so that it is expressed before M_1 in Minsoy. C – N_2 changes in Noir so that its period of expression is greatly extended.

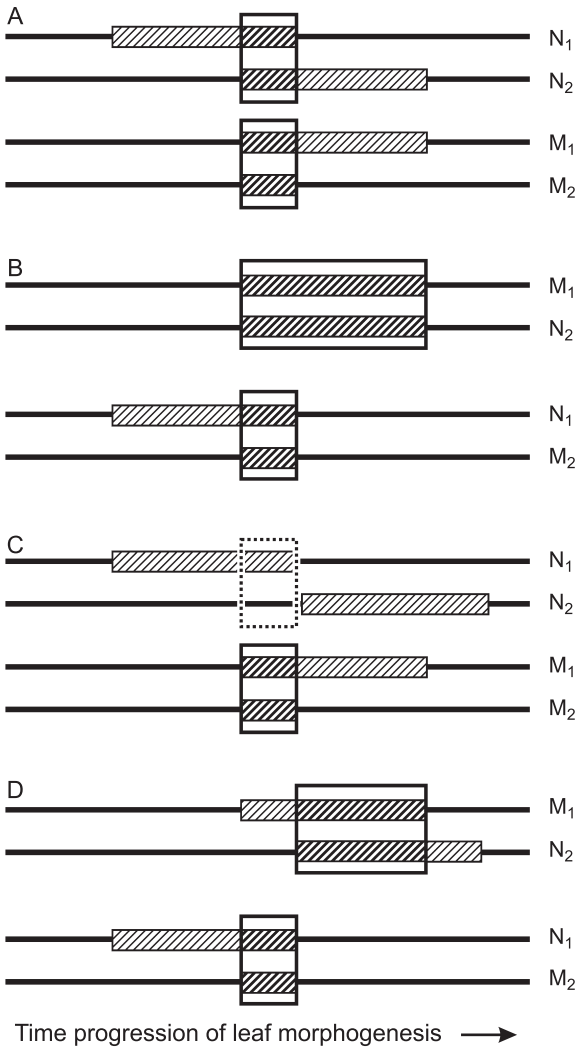

Fig. 5. MN RI segregants produced by crossing the parental lines in Figure 4 (C).

A, B – segregants grown in the same environment as the parents in Figure 4C. A – segregants with parental genotypes; B – segregants with mixed genotypes.

Assume that the frequency of multi-foliolate leaves is proportional to the period during which both locus1 and locus 2 are expressed (expressions of locus 1 & 2 overlap boxed). Whereas the frequency will be the same in the parental lines, one of the mixed genotypes will have a much higher frequency of multi-foliolates.

C, D – effect of environment on the genotypes in A and B. C – segregants with parental genotypes; D – segregants with mixed genotypes.

(Fig. 5B). The phenotypic pattern of the parental and mixed segregant genotypes in the RI population is similar to that which characterizes the interaction between U3 and U14-3 in the 2001 environment (Table 4). If interaction with the environment changes the period of expression of N_1N_2 (Fig. 5 C&D) a new phenotype characterizes this parental segregant genotype such that expression of N_1 and N_2 do not overlap and multi-foliolate leaves are not produced. This pattern corresponds to the segregation pattern seen in the interaction between U3 and U14 in the 2002 environment.

Conclusion

Three factors can contribute to the generation of genetic variation in inbred plants: Intrinsically variable mutations that occur with high frequency, are reversible and may have incremental effects on phenotypic functions. These have been demonstrated in vertebrates and insects, but are just beginning to be studied in plants (as advanced genomic techniques become available). By analogy with results for *Drosophila* these have great promise for producing the type of variation encountered when plants adapt to new environments.

Such mutations have a very large target in the form of somatic cells any of which can produce flowers and seed as well as the entire plant genome that is inherited without disruption, because of inbreeding.

The inheritance of the genome without disruption by outcrossing, allows the plant to develop a vast system of combinatorial interactions. We have presented examples of this taken from soybean. More evidence will certainly become available as genomics of other plants become available.

We live in an exciting period for biology and evolutionary science. As genomics information from plants and animals becomes available we can look forward to new insights into many of the questions that intrigued Dimitriy Belyaev as well as the discovery of new questions and puzzles that would have delighted him.

We imagine that environment selectively delays expression of N_2. The segregants with mixed phenotype remain for the most part unchanged M_1N_2 has a high frequency of multi-foliolates and N_1M_2 has a moderate to low frequency. However, the parental genotype N1N2 has very few or none.

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