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Cloning quantitative trait loci by insertional mutagenesis*

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Summary. This study explores the theoretical potential of "insertional mutagenesis" (i.e., mutagenesis as a result of integration of novel DNA sequences into the germ line), as a means of cloning quantitative trait loci (QTL). The approach presented is based on a direct search for mutagenic effects of a quantitative nature, and makes no assumptions as to the nature of the loci affecting quantitative trait value. Since there are a very large number of potential insertion sites in the genome but only a limited number of target sites that can affect any particular trait, large numbers of inserts will have to be generated and screened. The effects of allelic variants at any single QTL on phenotype value are expected to be small relative to sampling variation. Thus two of three stages of replicate testing will be required for each insert in order to bring overall Type I error down to negligible proportions and yet maintain good statistical power for inserts with true effects on the quantitative traits under consideration. The overall effort involved will depend on the spectrum of mutagenic effects produced by insertional mutagenesis. This spectrum is presently unknown, but using reasonable estimates, about 10,000 inserts would have to be tested, at reasonable replicate numbers ($n \geqq 30$) and Type I error ($\alpha = 0.01$) in the first testing stage, to provide a high likelihood of detecting at least one insert with a true effect on a given quantitative trait of interest. Total offspring numbers required per true quantitative mutagenic effect detected decrease strongly with increased number of traits scored and increased number of inserts per initial transformed parent. In fact, it would appear that successful implementation of experiments of this sort will require the introduction of multiple independent inserts in the original parent individuals, by means of repeated transformation, or use of transposable elements as inserts. When biologically feasible, selfing would appear to be the method of choice for insert replication, and in all cases the experiments must be carried out in inbred lines to reduce error variation due to genetic segregation, and avoid confounding mutational effects of the insert with effects due to linkage with nearby segregating QTL. The special qualifications of *Arabidopsis thaliana* for studies of this sort are emphasized, and problems raised by somaclonal variation are discussed.

Key words: Insertional mutagenesis – Quantitative trait loci – Cloning

Introduction

Most traits of agricultural importance are polygenic and quantitative in nature, i.e., genetic variation in the trait is dependent on allelic differences at a number of loci, each having relatively small effects on phenotypic value. Trait value is more or less strongly affected by environmental variables. Polygenic loci which affect quantitative traits (henceforth, quantitative trait loci, QTL) have neither known protein products nor striking phenotypic effects. Thus they have hitherto not been amenable to the usual cloning procedures. In this study we explore, at a theoretical level, the potential of "insertional mutagenesis", i.e., mutagenesis as a result of integration of novel DNA sequences into the germ line, as a means of cloning quantitative trait loci.

Insertional mutagenesis was first demonstrated in maize (McClintock 1984), and has since been docu-

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mented in prokaryotes (Shapiro 1983), yeast (Eibel and Phillippsen 1984), *Drosophila* (Swaroop et al. 1985), mouse (Jenkins et al. 1981) and snapdragon (Bonas et al. 1984). Experimentally, insertional mutagenesis has been observed as a consequence of DNA insertions which are spontaneous (Bonas et al. 1984; Jenkins et al. 1981), induced (Johns et al. 1985; McClintock 1984) or engineered (Andre et al. 1986; Jaenisch et al. 1983; Palmiter et al. 1984; Wagner et al. 1983). Sites of insertion appear to be distributed more or less at random (e.g. Goldberg et al. 1983; Lacy et al. 1983; Nelson et al. 1984; Tunnacliffe et al. 1983), although there is some evidence for specificity of the exact point of integration (O'Hare and Rubin 1983). Thus, DNA insertion can apparently be a potent and general mutagen.

The unique attribute of insertional mutagenesis the use of the inserted sequence for retrieving and cloning the affected normal gene. This is illustrated by the cloning of the "white eye" locus of *Drosophila* (Bingham et al. 1981) and the α(I) collagen gene in mice (Jaenisch of integration (O'Hare and Rubin 1983). Thus, DNA appear to be quite general (Beckmann and Soller 1985, 1986a, b) and with appropriate modification, ought to allow the cloning of polygenes affecting quantitative traits as well. That QTL are subject to mutagenesis is well established (Bathai and Swaminathan 1962; Joshi and Frey 1969; Lawrence 1968; Mackay 1984, 1985; Scossiroli et al. 1966). Indeed, Mackay (1984, 1985) has recently induced polygenic variation in bristle number in *Drosophila* by making use of P-M hybrid dysgenesis to induce P-element mobility and has shown that the induced variation could be exploited to produce lines with both high and low bristle numbers. Mackay (1984, 1985) specifically pointed out that the high and low lines could eventually be used to retrieve the polygenic loci involved in the response to selection.

There are, however, two basic problems in the use of insertional mutagenesis to clone QTL. The first is that there is a very large number of potential insertion sites in the genome, but only a limited number of target sites than can affect any particular trait. Thus, very large numbers of inserts will have to be generated and screened to obtain mutants affecting a specific trait. The second problem is that the effects of allelic variants at any single QTL on phenotype value are indistinguishable from the effects of other loci or of environmental factors affecting the given trait. Thus it will be necessary to use special experimental designs to identify carriers of mutagenized QTL affecting a particular trait. One possibility, appropriate for species with highly inbred lines available, is to test each insert in replicate, to allow the effects of the insert to be distinguished from the effects of background variation. This approach will be discussed in detail in the present paper. Since among agricultural species, highly inbred lines are virtually limited to plants, the discussion will be carried out in terms of plant species.

Theory

Total number of inserts required

On average, a sample of inserts of size N would be expected to provide $\theta = NL(1-\beta)$ detected inserts truly affecting a particular quantitative trait, where L is the proportion of inserts affecting the trait, $(1-\beta)$ the power of the experiment and β the likelihood of Type II error i.e., the likelihood that an insert of interest, although present in the sample, is not detected. β will depend on various design and biological parameters, as will be discussed in the following sections. Assuming, as documented above, that an inserted sequence will distribute in a random or quasi-random manner across the genome, $L = kwG/B$, where

- G = total target size of a gene, including exons, introns, contiguous control regions and adjacent upstream and downstream regions in which an insert can affect gene function;
- w = the proportion of inserts in the target area of a gene that will affect gene function in a detectable manner;
- B = total genome size;
- k = the total number of loci potentially affecting the particular trait in question.

The number of detected inserts with true effects in a sample of size N will have a Poisson distribution, with Poisson parameter, θ, defined as above. It seems reasonable to aim at a value of $\theta = 2.0$, which gives a likelihood of 0.87 that a given sample will provide at least one detected insert with a true effect (Walpole and Myers 1978). Thus, the total number of inserts tested should be on the order of $N = 2/L(1-\beta)$. In most cases, detecting inserts of interest will involve a two- or even three-stage experiment. In this case, the denominator of the expression for N will include the product of the power terms for the various stages of the experiment, e.g. $(1-\beta_1)(1-\beta_2)$, for a two-stage experiment. Under replicated testing, individuals can be scored simultaneously for any desired number, t, of quantitative traits. In this case, the expected number of inserts detected as having an effect on one or another of the traits will equal $NtL(1-\beta)$.

Power of the experiment

Testing an insert in replicate can be carried out by (1) vegetative reproduction of the original heterozygous transformant; (2) selfing or dihaploidizing the original

Table 1. Genotype composition and mean value of replicated insert populations according to mode of replication and dominance status of insert

Mode of replication	Genotype composition quantitative value[1]			Mean value[2]	Mean value relative to selfing mode[3] according to dominance status of insert		
	II 2d	Ii d+h	ii 0		Recessive	Codominant	Dominant
Selfing							
S-1	1/4	1/2	1/4	(d+h)/2	1/2	1	3/2
S-4[4]	1/2	0	1/2	d	1	1	1
Vegetative		1		h	0	1	2
Crossing two independent transformants							
F-1		1/2		h/2	0	1/2	1
F-2	1/16	6/16	9/16	(d+3h)/8	1/8	1/2	7/8

[1] Letting I represent presence of the insert and i its absence
[2] See text for details and for definitions of d and h
[3] Compared with selfing-mode, codominance case
[4] Equivalent to diploidization

transformant and scoring the S-1 or subsequent selfed generations or (3) crossing two independent transformants and intercrossing the F-1 products to produce an F-2 population, scoring both the F-1 and F-2 products. The effect, D, of the insert on the mean quantitative trait value of the replicated population will differ in each case, depending on the proportion of various insert genotypes in the replicated population. For the sake of generality, D will be "standardized", i.e. defined in units of the phenotypic standard deviation of the trait in question, and will be a function of

2d, the "standardized" effect of the insert on quantitative value in the homozygote (II),

d+h, the "standardized" effect of the insert on quantitative value in the heterozygote (Ii),

both expressed as a deviation from the value of the homozygous null genotype (ii),

Quantitative value	0 ↓	d+h ↓	+2d ↓
Genotype	(ii)	(Ii)	(II)

Table 1 shows the genotype composition and mean value of the replicated insert population, as a function of d and h, by mode of replication and dominance status of the insert. Except for the case of complete dominance, the insert has the greatest effect on the replicated population in the selfing mode. For the crossing mode, the insert effect is generally half that of the selfing mode. With complete dominance, a greater effect is obtained with vegetative reproduction, but the advantage is not large. Thus, when biologically feasible, selfing would appear to be the method of choice for insert replication. When selfing or crossing modes are utilized, it is assumed that the initial parental transformants were taken from a genetically uniform, homozygous population, so that genetic sources of variation, aside from the inserts themselves, are not present in the offspring. This restriction would not apply for vegetative replication.

Given a replicated population of inserts, the likelihood of a Type II error, β, will equal the integral of the normal standard curve from $-\infty$ to x_{β_1} where

$x_\beta = x_{\alpha/2} - n^{1/2} D_1$,

$x_{\alpha/2}$ = the abscissa of the standardized normal curve such that the area under the curve from $-\infty$ to $x_{\alpha/2}$ is equal to $\alpha/2$,

α = the two-tail likelihood of a Type I error (i.e. of obtaining a significant effect on mean offspring value by chance alone), and

n = the number of replicates tested per insert.

Note that the replication numbers required for a given power will be inversely proportional to D^2. Thus, for obligatory outcrossers, where a crossing mode of replication must be utilized, the number of replicates required for given power will be upwards of twice that for selfers, depending on the dominance status of the insert effect (Table 1).

Total number of individuals (offspring) scored for the quantitative traits

The total number of individuals scored for quantitative traits will be proportional to the product of the total number of inserts tested, N, and the number of replicates scored per insert, n. Significant savings in the total numbers scored for the quantitative traits can be achieved by carrying out multiple insertions in the

same parent individual, so that a single offspring group will be segregating for a number, m, of independent inserts. In this way, each offspring group scored for quantitative traits will serve to evaluate the effects of all m inserts. Each insert can be followed as a distinct, co-dominant genetic entity, independent of any phenotypic effect it might have, since it will form a restriction fragment length polymorphism (RFLP) at the site of insertion, which can be detected using the insert as a probe (Beckmann and Soller 1985, 1986a). Alternatively, the inserts could be designed to confer an easily scorable marker trait. The m inserts of any offspring group that showed significant effects on a quantitative trait would be followed in backcross or F-2 generations, and tested individually for their effects by comparing the mean quantitative value of their respective II, Ii and ii genotypes. Only the insert affecting the trait should show significant differences between insert genotypes.

Since a large number of inserts will be tested against one or more quantitative traits, a greater or lesser number of "false" effects will be detected, depending on the magnitude set for Type I error. Eliminating these false effects will require a second or even third stage of testing, until the expected number of retained "false" effects is below some predetermined number. The total number, T, of offspring scored for the quantitative traits will thus be equal to the sum of the number scored in each of the testing stages, as follows:

$$T = \sum_{i=1}^{3} T_i$$

where,

$T_1 = Nn_1/m$,
$T_2 = Nn_2\,[L(1-\beta_2)+\alpha_1/m]$, and
$T_3 = Nn_3\,[L(1-\beta_1)(1-\beta_2)+\alpha_1\alpha_2/m]$

where all symbols are as defined previously and the subscripts refer to the first, second or third testing stage, respectively.

Numerical results

Proportion of inserts affecting a quantitative trait

In calculating the proportion (L) of inserts expected to affect a quantitative trait, the only known parameter is total genome size (B). All of the other parameters, namely, the effective target size of the gene (G), the proportion of inserts in the target area that affect gene function (w) and the number of loci potentially affecting quantitative trait value (k) can be assigned values only within fairly wide limits. In Table 2 a range of estimates is presented for L using conservative, temperate and optimistic estimates of the above parameters. Estimates are presented for *Tabacum,* which has a haploid genome of $B = 1.6 \times 10^9$ bp, fairly typical for a diploid higher plant, and for *Arabidopsis thaliana,* a dicotyledenous plant having a remarkably small genome of $B = 7 \times 10^7$ bp (Leutwiler et al. 1984; Somerville et al. 1985). Estimates for L range from approximately 1/125,000 to 1/1,250 for *Tabacum,* and from 1/12,000 to 1/600 for *Arabidopsis.* If more than one quantitative trait is scored, the likelihood of an insert affecting any one of them would increase in proportion. In the calculations that follow, an estimate of L = 1/3,000 will be used, which is temperate to optimistic for *Tabacum,* and temperate to conservative for *Arabidopsis.*

Power of the test

The power of the replicate-test design as a function of the number of replicates, *n*, was calculated for the selfing mode of replication. For the other replication modes, the number of replicates required for a given power will stand in inverse proportion to the squared effect of the insert on mean population value relative to the selfing mode as given in Table 1.

The calculations that follow assume an insert having a mean effect of D = 0.5. This corresponds to an effect of $2d = +1.0\,\sigma$ for the insert in homozygous condition, and an effect of h = 0 (lack of dominance) for the insert in heterozygous condition. Although this is greater than most biometrical estimates of allelic effects at QTL, the latter are based on naturally occurring genetic variation remaining after the screening effects of natural and artificial selection. It is likely that the original spectrum of mutagenic effects includes larger effects. For example, in barley, extensive screening for erectoid mutants showed that most mutants, when homozygous, had effects considerably greater than $1.0\,\sigma$ (Persson and Hagberg 1969). In addition, biometrical estimates are generally made with respect to the total phenotypic variation, including both genetic and environmental sources, while the proposed studies will be carried out within genetically uniform populations. Consequently, insert effects will be measured relative to a smaller base unit than is customary in biometrical studies.

Table 3 shows the power of the replicate test with respect to an insert having an effect of the above magnitude, as a function of Type I error, α, and replicate number, *n*. At moderate replicate numbers ($n = 20$–50), there is a sharp increase in power as α increases from 0.001 to 0.01 or 0.05, but only a small additional increase in power as α increases further to 0.10. At low replicate numbers ($n = 10$), power is low at all values of α. The converse is true when $n = 100$.

Table 2. Proportion (L) of inserts affecting a quantitative trait on conservative, temperate and optimistic assumptions, for a normal-size (*Tabacum*) plant genome and the small *Arabidopsis* genome

Parameter	*Tabacum*			*Arabidopsis*		
	Conservative	Temperate	Optimistic	Conservative	Temperate	Optimistic
Gene size (G) in kb	4	10	20		1.2[1]	
Genome size (B) in kb		1.6×10^6			7×10^4	
Proportion of inserts affecting gene function (w)						
	0.5	0.8	1.0	0.5	0.8	1.0
No. of loci affecting a quantitative trait (k)						
	10	50	100	10	50	100
L = kvG/B	1/125,000	1/25,000	1/1,250	1/11,650	1/1,450	1/600

[1] Assuming total genome size of 70,000 kb, 12% repetitive DNA and total number of genes of 50,000

Number of inserts tested

Table 3 also shows that about 10,000 inserts must be tested, at reasonable values of n ($\geqq 30$) and α ($\geqq 0.01$) in the first testing stage, to provide high likelihood of detecting at least one insert with an effect on a given quantitative trait of interest. This will be the same for all higher plants and animals, given a similar density of target sites per 10^6 bp. It is assumed that testing will be carried out until the expected number of "false" effects is less than 1.0. It is also assumed that when a second round of testing is required, it will be carried out at $\alpha = 0.01$, $n = 50$, to give power of 0.83, and that a third round of testing, if necessary, will be carried out at $\alpha = 0.01$, $n = 100$, to give a power of 0.99. If more than one quantitative trait is scored, the number of inserts that must be tested to detect at least one insert with a true effect on any of the traits decreases proportionately, i.e. if ten independent traits were scored, it would be necessary to test only about 1,000 inserts to be reasonably confident of detecting at least one insert truly affecting one of the traits under consideration.

Table 3. Power of the replicate test design as a function of replicate number and Type I error (above) and total number of inserts tested (in thousands) to provide a reasonable likelihood of detecting at least one insert with a true effect on the quantitative trait of interest[1] (below)

No. of replicates (n)	Type I error (α)				
	0.001	0.01	0.05	0.10	0.15
5	0.02	0.07	0.20	0.30	0.37
	361.4	103.3	36.1	24.1	19.5
10	0.04	0.16	0.35	0.47	0.56
	180.7	45.2	20.6	15.4	12.9
20	0.15	0.37	0.61	0.72	0.78
	48.2	19.5	11.8	10.0	9.3
30	0.29	0.57	0.78	0.86	0.90
	24.9	12.7	9.3	8.4	8.0
50	0.60	0.83	0.94	0.97	0.98
	12.0	8.7	7.7	7.4	7.4
100	0.96	0.99	1.00	1.00	1.00
	7.5	7.3	7.2	7.2	7.2

[1] Assumed insert effect, D = 0.5; see text for details

Total number of offspring evaluated

With a single insert per parent plant, minimum numbers (Table 4) are obtained with rather low replicate numbers ($n = 10$) and high Type I error ($\alpha = 0.15$). With five inserts per parent plant, the minimum moves to $n = 20$ and $\alpha = 0.05$, and with ten inserts per parent plant the minimum is at $n = 30$ and $\alpha = 0.01$. Total offspring numbers seem prohibitively large for m = 1 and it would appear that successful implementation of experiments of this sort requires the introduction of multiple independent inserts in the original parent plants.

If more than one quantitative trait is scored, the number of offspring that must be raised in order to detect an insert with an effect on any one of the traits decreases proportionately, e.g. if ten traits were scored, and the original transformants carried an average of 10 inserts each, it would be necessary to raise and score only some 5,000 offspring plants, to detect an insert with an effect on one or other of the traits under consideration. Note that for an obligatory outcrosser (crossing mode of replication), 2 to 16 times as many plants would have to be scored, depending on the dominance status of the insert.

Table 4. Total number of offspring (in thousands) raised in the replicate-test design in order to detect an effect on a given trait as a function of number of replicates, Type I error and number of inserts per parent plant[1]

No. of replicates	No. of inserts per parent	Type I error				
		0.001 ($\times 10^{-3}$)	0.01 ($\times 10^{-3}$)	0.05 ($\times 10^{-3}$)	0.10 ($\times 10^{-3}$)	0.15 ($\times 10^{-3}$)
5	1	3,633.2	1,085.7	435.9	364.2	345.2
	5	380.2	156.3	128.6	147.3	169.3
	10	199.5	104.6	110.6	135.3	159.6
10	1	1,816.7	475.2	259.5	232.6	228.2
	5	371.0	113.7	94.3	107.7	124.9
	10	190.3	68.5	73.6	94.1	112.0
20	1	966.6	400.9	267.5	252.3	256.6
	5	195.5	88.4	77.9	91.7	108.3
	10	99.1	49.3	54.2	71.6	89.8
30	1	749.2	381.4	302.1	295.2	302.7
	5	151.1	82.9	79.6	93.6	93.9
	10	76.4	44.8	51.8	68.4	85.9
50	1	603.4	440.3	404.4	410.8	425.6
	5	121.4	91.9	96.8	112.8	130.5
	10	61.2	48.3	58.4	75.6	97.4
100	1	753.7	734.0	741.8	760.2	778.5
	5	151.3	150.0	163.4	181.8	200.2
	10	76.0	77.0	91.1	109.5	127.9

[1] Assumed insert effect, D=0.5; see text for details. It is assumed that three stages of selection are employed, with replicate numbers for the second and third stages equal to 50 and 100, respectively, and Type I error in both cases set at 0.01

Discussion

The results of this study show that a programme involving large numbers of independent insertions, followed by testing the quantitative phenotypic effect of each insert in replicate, may represent an experimentally feasible approach for the identification and cloning of chromosomal regions at which insertion of a foreign DNA sequence has affected quantitative traits. The approach presented here is based on a direct search for mutagenic effects of a quantitative nature, and makes no assumptions as to the nature of the loci affecting quantitative trait value. In this it differs from the approach presented by Robertson (1985), which is based on the somewhat controversial proposition that most quantitative trait alleles represent wild-type isoalleles at loci producing major effects on quantitative phenotype. Robertson (1985) therefore suggested that loci which have major effects on the quantitative phenotype be cloned by means of insertional mutagenesis. He also suggested that wild-type alleles at these loci – isolated by screening appropriate genomic libraries, and identified as such by direct sequencing or RFLP methodologies – be tested for quantitative effects.

The experimental design proposed here is straightforward but the effort involved will depend on the values of a number of basic biotechnical parameters, including: the number of potential regions at which an inserted DNA sequence can affect quantitative trait value, the likelihood that an insert in the region will in fact affect trait value and the magnitude of effect on trait value. The range of parameter values assumed in this analysis implies that a great many inserts will have to be tested to detect even one insert affecting any one of a large number of quantitative traits. That the quest is far from hopeless, however, is indicated by the numerous mutagenic treatments found to affect quantitative traits in a wide variety of organisms, while a relatively high proportion of inserts in mice have major phenotypic effects (Covarrubias et al. 1986; Palmiter and Brinster 1986). In the final analysis, validation of the approach described here will depend on the experimental determination of the spectrum of mutagenic effects produced by insertional mutagenesis.

Brute-force vs cleverness

The approach to identification of mutations affecting quantitative trait loci proposed in this study is based on replicating each insert many times. In some instances, special screening methods can be used in which small quantitative effects in the trait of interest are translated

into large qualitative effects on phenotype. A notable instance was the screening for internode length mutants in barley as erectoid or lax phenotypes (Persson and Hagberg 1969). Additional examples might be flowering time or fruit ripening, in which small differences in maturation time are translated into large differences in flower or fruit appearance. Other examples are traits such as growth rate under crowded conditions, in which small quantitative differences are translated into a large competitive advantage that eventually results in large phenotypic differences.

Replicate testing in obligatory outcrossers and segregating populations

Obligatory outcrossers, even when fully inbred lines are available (e.g. in mice or corn), will require a crossing mode of replication. The effect of any given insert on mean F-1 or F-2 value of the offspring population will be small compared to the selfing mode. This will involve a correspondingly large increase in the number of offspring per family group. Although we have not investigated this in detail, exploratory calculations show that it would be most effective to spread the replications over time, carrying out two or three generations of selection for the quantitative trait of interest within the offspring population produced by crossing the initial transformants. This would rapidly increase frequencies of an insert affecting the trait under selection to the magnitude postulated in this study. Indeed, depending on the intensity of selection, after two generations of selection, frequencies for such inserts might be at about the same level as those expected under a selfing mode of replication, and could be evaluated accordingly. The main disadvantage of this procedure is the additional number of offspring that must be evaluated during the selection generations. Although in theory one could carry out index selection for a number of traits simultaneously, this would lower the rate of response to selection for inserts affecting any one trait, and would lengthen the number of generations required for the selection step. Consequently, the intercalation of a selection step basically limits the procedure to a single trait of interest. Selection, followed by replicate testing, would, however, be appropriate for traits such as polygenic resistance to stressors, where convenient means of screening large populations are available, and where a single trait is of paramount interest.

As an alternative to evaluating the effect of an insert on mean trait value as a means of identifying mutagenic effects, one could look for an increase in the within-family variance produced by segregation of an insert affecting the quantitative trait. Here, too, very large numbers would be required to detect an increase in variance without loss in experimental power. One could also follow each insert independently in the offspring population, and compare the quantitative value of individuals which are homozygous and heterozygous for the insert with homozygous nulls. This would require increased numbers of offspring per family group and would also require screening each individual for the presence of the insert, a formidable task if insert presence is detected by RFLP patterns, but feasible if the insert carried a readily scorable marker. All in all, though, for obligatory outcrossers, testing in replicate would require very large numbers of offspring and hence does not seem to be a feasible means of identifying inserts that have affected quantitative traits, except perhaps for polygenic resistance traits.

In the programmes considered until this point, it has been assumed that fully inbred lines are available for the initiation of the transformation programme. If such lines are not available, it will not be possible, using a replicate design, to distinguish between effects due to the inserts and those due to residual genetic variation distinguishing a particular family from others in the population. Also, in a segregating population, an insert associated quantitative effect may result from close linkage of the insert to a polymorphic QTL, rather than mutagenesis. Thus, the replicate design will be useful when dealing with fully inbred lines and species that tolerate selfing.

Multiple inserts

A major conclusion of this study is the importance of obtaining multiple inserts in each transformed individual in order to bring experimental numbers to reasonable magnitudes. This could be achieved, for example, by sequential or simultaneous transformation of the same plant using inserts carrying different selectable or scorable markers. Experience with transformation of plants by means of the Ti plasmid of *Agrobacterium tumefaciens* indicates that successful transformation can involve multiple independent insertions into the genome (Annick et al. 1986; Ursic et al. 1983) and that repeated transformation of the same plant is possible. A different approach would be to introduce a transposable element into the recipient genome which could be mobilized upon suitable stimulus, or to mobilize an existing transposon as exemplified by Mackay (1984, 1985).

Natural transposons as mutagenizing inserts

An alternative approach to achieving muliple inserts per individual is to search for quantitative trait variation associated with insertion sites of naturally existing transposons, as these are identified and cloned. This assumes that existing transposon insertions in the ge-

nome are a significant source of quantitative genetic variation in the population. To be effective, the population must be segregating for the transposon insertion site of interest, or populations differing in their insertion site pattern for the same transposon must be available. Such populations could be crossed. In addition, associations of particular insertion sites with quantitative traits could be investigated by treating the inserts as RFLP type markers and using general techniques for marker-based analyses of quantitative traits (Beckmann and Soller 1986a; Soller and Beckmann 1983; Soller et al. 1976) to look for markers with pleiotropic effects on quantitative traits. This approach might be particularly effective in populations subjected to long-term selection for particular quantitative traits. To the extent that transposon-mediated insertional mutagenesis is a source of novel genetic variation in such populations, any transposon insertions with positive effects on the trait under selection could be expected to reach high frequencies. Consequently, those found at high frequencies would be candidates for further screening as putative sources of loci with quantitative effects on the trait under selection. One possibility for such a study might be the high and low oil- and protein-percentage selection lines in maize, which give a consistent response to selection for over 75 generations (Dudley 1977). Transposon insertions which differ in the two selection lines might be good candidates for further screening for quantitative effects on the selection trait. Similarly, insert sites of endogenous viruses in poultry have been found to differ between layer populations (E. Smith, pers. commun; unpublished data our laboratory) and between layers and heavy breeds (S. Hughes, pers. commun.). An endogenous virus insertion is apparently the cause of a mutation in the Dilute (d) coat colour locus of mice (Copeland et al. 1983; Jenkins et al. 1981). Thus, endogenous viruses at high frequencies in specific populations, but which differ between populations, would be candidates for investigation as mutagenizing inserts for quantitative traits.

Genome size

To a certain extent, the so-called "non-functional"DNA in the genome (e.g. spacers and repetitive sequences) can be expected to act as an insert "sink" in the sense that inserts into these regions may have little effect on gene function and phenotype. For this reason, the proportion of "non-functional" DNA in the genome can be expected to have a major effect on the total number of inserts required in an insertional mutagenesis programme. In the usual plant species, such DNA comprises as much as 80% of the genome. Thus, four out of five inserts can be expected to have little effect on phenotype. From this point of wiew the remarkably small genome size and the relative lack of repetitive DNA in *Arabidopsis thaliana* (Leutwiler et al. 1984) provide a unique advantage for an insertional mutagenesis programme. Since virtually all of the genome in this species comprises coding or regulatory sequences, the majority of inserts are probably overtly mutagenic.

An alternative possibility is to use some means of screening for inserts that have interrupted functional DNA regions. For example, as shown by Andre et al. (1986) if the insert contains a selectable marker lacking a promotor, then only inserts that enter the genome near a promotor would show the marker phenotype. Screening for the marker phenotype would thus enable ready selection for inserts that are in functional regions.

Somaclonal variation

At present, transformation of dicotyledenous plants is generally achieved by using the Ti plasmid of *Agrobacterium tumefaciens* and involves a tissue-culture step. It induces wide-ranging mutations in numerous plant species. This phenomenon is termed "somaclonal variation" and has generated considerable interest in plant-breeding circles as a powerful means of mutagenesis (Snowcroft 1985). Somaclonal variation, however, will seriously interfere with the identification of inserts associated with quantitative trait effects. Estimates of the number of somaclonally-induced mutational events per regenerated plant, based on the observed number of Mendelian mutations induced (see e.g. Evans and Sharp 1983), suggest that each regenerant undergoes on the order of ten independent mutational events in coding regions. This number is one order of magnitude greater than that expected for insertional mutagenesis, but is small enough for only a low likelihood of close linkage between an insert and a somaclonal mutant. Thus, the somaclonal mutants may be eliminated by carrying out a series of three to five backcrosses to the normal parent, retaining the inserts in each generation by way of their selectable or scorable markers. Only when the backcrossing procedure is completed would the transformants be replicated and inserts evaluated for quantitative or other effects on phenotype.

A better means of avoiding the problems introduced by somaclonal variation would be to insert a transposable element in which the frequency of transposition can be regulated to use for transformation of the material in which insertional mutagenesis is induced. After initial transformation with the constructed element, any induced somaclonal mutants could be eliminated by backcrosses, as above, retaining the insert by way of its RFLP pattern or a suitable marker. The transformants could then be multipled by gametic or

vegetative reproduction to provide a large population of individuals containing the insert. If the insert is then induced to transpose, a large number of independent insertions would be produced without the possibility of somaclonal variation.

Interpreting and exploiting cloned QTL

Once an insert affecting a quantitative trait is identified, the next step will be to rescue the normal sequence at the point of insertion. The normal sequence, once cloned, could be tested in turn for quantitative effects by reinsertion in single or multiple copies, or with modified control elements. In theory, the normal homologue of the mutated putative QTL could be reinserted in the mutant individual itself, to test for complementation. In addition, the cloned QTL would be sequenced and examined for open reading frames, promoter or enhancer sequences, etc. It would also be possible to "walk" up and down the genome from the point of insertion, to determine the presence of coding or other sequences of interest in the vicinity of the insertion. It can be expected that as information accumulates on the nature of the sequences associated with insertional effects of a quantitative nature, understanding will increase on the molecular and, eventually, the biochemical and physiological nature of loci affecting quantitative traits. This may eventually resolve the controversy as to whether genetic elements involved in quantitative genetic variation are pleiotropic effects of major gene mutations, isoalleles of major genes or a special class of genetic element, different in kind from the loci giving rise to major effects on phenotype.

Given the interrelated nature of much plant and animal physiology, it is possible that cloning even one or two loci in a polygenic system will enable a large degree of genetic manipulation of trait value, e.g. by reinsertion in single or multiple copies or with modified control sequences. That is, forcing one function involved in the expression of a polygenic quantitative trait to high levels may cause the entire system to adjust proportionately. This is observed, for example, in the growth-hormone-transformed mice described by Palmiter et al. (1983). Thus, for many scientific and applied purposes, it may be sufficient to clone only a few of the many polygenic loci affecting any particular quantitative trait.

In this context it is useful to recall that there is apparently a considerable amount of sequence homology between loci affecting similar traits, even across widely separated groups. Cloned human genes, for example, are routinely used as probes to screen for their counterparts in other mammalian species. Hence loci cloned in one organism might be used to retrieve the corresponding loci in other species of the same general taxonomic grouping, and by judicious "genome-hopping" in more remote taxonomic groupings as well. Thus, it might not always be necessary to repeat the insertional mutagenesis cloning procedure separately for each agricultural species. One might start with *Arabidopsis* or mouse and end up with sunflower or cows.

Conclusions

The purpose of this study has been to identify and evaluate, quantitatively when possible, some of the issues determining the feasibility of cloning genes of economic value by means of insertional mutagenesis. The overall impression is that severe obstacles remain to be overcome, particularly somaclonal variation, the necessity for multiple inserts and the necessity of screening large numbers of individuals. However, the goal does not seem hopeless. The use of *Arabidopsis thalaiana* as an experimental organism may increase the likelihood of obtaining quantitative effects. Technical solutions have been proposed for some of the problems; others will surely surface in time. Much will also depend on the actual values for some of the biological parameters involved. Information is most lacking on the actual spectrum of mutagenic effects on the phenotype, including quantitative effects. In view of the enormous scientific and practical importance of cloning loci having quantitative effects on traits of economic importance, experimental studies in this area would appear to be amply justified.

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