Dissection of a Synthesized Quantitative Trait to Characterize Transgene Interactions

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ABSTRACT

Six transgenic tobacco lines, each homozygous for the β -glucuronidase (GUS) gene at a different locus, and wild type were selfed and intercrossed to evaluate GUS activity in all possible hemizygous, homozygous and dihybrid combinations of GUS alleles. The transgenic lines are characterized by their GUS activity (two low, three intermediate, one high), T-DNA complexity (four single-copy, two more complex single-locus) and the presence of the chicken lysozyme matrix-associated region (MAR) around the full T-DNA (two lines). Gene action and interaction was analyzed by weighted linear regression with parameters for additivity, dominance and epistasis. The analysis showed that each of the four single-copy lines acted fully additively. In contrast, the two complex single-locus lines showed classical single-locus overdominance and were epistatic dominant over all other GUS alleles. The latter is manifested in severe suppression of GUS activity in dihybrid lines, irrespective of the presence of MAR elements around the GUS gene. Such elements apparently do not protect against epistatic dominance. The quantitative data suggested that the epistatic dominance and overdominance are based on the same molecular mechanism. Our approach of a genetic analysis of quantitative variation in well-characterized transgenic lines provides a powerful tool to gain insight into complex plant traits.

THE performance of transgenic plants after genetic modification is less predictable than often desired for scientific or commercial applications. In addition to large variation in the levels of expression of the newly introduced transgene (so-called position effects; PEACH and VELTEN 1991; MLYNÁROVÁ *et al.* 1994, 1995), a variety of generally undesired gene silencing phenomena and pleiotropic effects can occur (HOBBS *et al.* 1990, 1993; MATZKE and MATZKE 1993; FLAVELL 1994; FINNEGAN and McELROY 1994; PASKOWSKI 1994; MEYER 1995b). A difference between desired and actual phenotype of a transgenic plant can become apparent soon after genetic transformation or may only become evident when agronomic performance is being assessed in the field (BRANDLE *et al.* 1995). As a consequence, the transgenic plant with the desired magnitude and stability of transgene expression has to be selected from a large population of independently derived transgenic plants (CONNER and CHRISTEY 1994). This situation is not very different from the selection for yield and stability, or any other trait of interest, in plant breeding (BOS and CALIGARI 1995). Genetics and breeding are likely to face the pyramiding of favorable (trans)genes. Understanding and controlling the way genes interact will

be essential for the success of manipulating complex traits (PATTERSON and TODD 1995). In recent years, considerable progress has been made in detecting loci encoding complex traits (quantitative trait loci or QTLs; JANSEN 1996). The next challenge in this endeavor will be to isolate the corresponding genes and to study how they interact to result in the particular phenotype (FRANKEL and SCHORK 1996). To meet this challenge, transgenic plants can be of help because they offer material in which the genetic constitution is precisely defined. We here synthesize a simple quantitative trait by combining different loci for the β -glucuronidase (GUS) reporter gene. Six independent transgenic tobacco (*Nicotiana tabacum* L.) plants, each homozygous for a single locus of GUS gene, and wild-type tobacco, the nulliplex plant line, were selfed and intercrossed in all combinations. This yielded all possible hemizygous (monohybrid), double hemizygous (dihybrid) and homozygous offspring. The GUS activity in these progeny populations was analyzed with a weighted linear regression model to assess the interactions of the alleles both within a locus (dominance) and between loci (epistasis). Previously we have shown that the sensitivity to position effects among tobacco transformants is reduced by flanking transgenes with matrix-associated region (MAR) elements (MLYNÁROVÁ *et al.* 1994–1996). Two of the transgenic tobacco lines chosen for analysis carry such MAR sequences at the borders of the T-DNA to investigate the behavior of MAR-contained T-DNA in hybrid offspring.

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MATERIALS AND METHODS

Plant material: Transgenic tobacco (*N. tabacum* cv. Petit Havana SR1) was obtained by Agrobacterium-mediated transformation with the binary T-DNA vectors pPPG and pLM previously described (MLYNÁROVÁ *et al.* 1994). These T-DNA vectors carry a kanamycin resistance gene for selection and a *Lhca3.St.1* promoter-GUS fusion, either with the chicken lysozyme A element at the borders of the T-DNA (pLM) or without any A element (pPPG). Plants having one locus of T-DNA were identified by a 3:1 segregation for kanamycin resistance in seeds obtained from the selfed primary transformants. Plants homozygous for the T-DNA were identified by the absence of segregation in selfed and backcrossed progeny of the next generation. The T-DNA copy number was determined by DNA gel blot analysis (MLYNÁROVÁ *et al.* 1994). The characteristics of the six transgenic parental lines chosen for analysis are given in Table 1. To facilitate identification throughout the text, the plant lines involved are named after the T-DNA configuration they carry. Plants carrying the pLM T-DNA are designated ANLGA, plants with the pPPG T-DNA are designated NLG. In this system, A represents the A element, N the kanamycin resistance gene, L the *Lhca3.St.1* promoter (NAP *et al.* 1993b), and G the β -glucuronidase gene. The seven parent plants chosen comprised untransformed, wild-type tobacco (WT) and six single-locus transformants: two one-copy pLM-transformed plants, ANLGA-13 and ANLGA-38; two one-copy pPPG-transformed plants, NLG-11 and NLG-41; and two complex one-locus pPPG-transformed plants, NLG-4 and NLG-47. Detailed DNA gel blot analysis of the NLG-4 and NLG-47 integrations showed the presence of a T-DNA locus more complex than a single integration (L. MLYNÁROVÁ, unpublished observations). The latter two lines exhibit a GUS activity that is relatively low compared with the average GUS activity of primary transformants (MLYNÁROVÁ *et al.* 1994).

Experimental design: Each of the 49 crosses required to establish all combinations was performed at least in triplicate using standard procedures (WERNSMAN and MATZINGER 1980). Seed capsules were harvested when they turned brown at the apex, and seeds from identical crosses were pooled and stored at room temperature. From all 49 combinations, >50 seeds were sown. Approximately 3 weeks after germination, 10 randomly picked seedlings were transferred to individual pots. Pots were randomized, and six randomly chosen plants from each cross were assayed for GUS activity ~7 weeks after the seedlings were planted out. To minimize variation due to environment, seedlings and plants were treated as uniformly as possible and grown in a fully climatized greenhouse with heating <18° and ventilation >24°.

Independent assortment of T-DNA loci: One F₁ plant from each cross was allowed to set self-pollinated seed. The F₂ seeds were used to determine linkage of the T-DNA loci involved by screening the progeny for segregation of kanamycin resistance. Seeds were sterilized in 70% ethanol for ~30 sec, rinsed in sterile water, then suspended in 0.15% soft agar containing quarter-strength MS salts (MURASHIGE and SKOOG 1962) with 50–200 mg/liter kanamycin. Seeds were plated on 0.8% agar plates with the same MS medium using a glass rod and incubated at 25° under cool-white fluorescent lamps. After ~10 days incubation, the numbers of green, kanamycin-resistant, and bleached, kanamycin-sensitive, seedlings were counted. In our hands, this method yields a fast and reliable estimate of resistance and sensitivity to kanamycin in segregating seedling populations.

GUS assays and quantitative analyses: Measurement of GUS activity in tobacco leaves was performed as described (NAP *et al.* 1992). Samples were harvested as single 9-mm diameter discs cut through similar positions of the lamina

TABLE 1

Plant lines used in this study

Plant	T-DNA	MAR	Integration	GUS activity
Wild type	None	NA ^a	NA	Zero
ANLGA-13	pLM	+	1 copy	Intermediate
ANLGA-38	pLM	+	1 copy	Intermediate
NLG-11	pPPG	–	1 copy	High
NLG-41	pPPG	–	1 copy	Intermediate
NLG-4	pPPG	–	1 locus	Low
NLG-47	pPPG	–	1 locus	Low

^a NA, not applicable.

from leaves of the same age and position. Statistical evaluations were performed with Genstat 5 (PAYNE *et al.* 1993).

RESULTS

A parsimonious regression model for GUS activity in the F₁ progeny: GUS activity in F₁ plants was analyzed with a linear regression model. Basic assumptions in such a regression analysis are additivity (in statistical sense) of the model terms, as well as independence and identical distribution of residual errors. When these requirements are not satisfied on the original scale of measurement, a transformation of the data is needed. Here, the standard deviation increases with the mean GUS activity, which indicates the need for a logarithmic transformation of GUS activity (see also NAP *et al.* 1993a; MLYNÁROVÁ *et al.* 1994). Although the quantitative analysis could easily be performed with logarithmically transformed data, genetic concepts such as additivity, dominance and epistasis fully depend on the scale chosen for analysis (MATHER and JINKS 1982). Moreover, such genetic concepts are often considered most appropriate on the scale of measurement. Therefore, our regression analysis was performed on the original scale of measurement using the geometric (*i.e.*, backtransformed logarithmic) mean of the GUS activities of the replicates assayed. To take the different variances within different progenies into account, a weight factor was introduced that is inversely proportional to the variance. This weight factor is based on the following: if the mean μ of n values is normally distributed as $N(\mu, \sigma^2/n)$ on the logarithmic scale, the backtransformed mean e^μ is approximately normally distributed as $N(e^\mu, e^{2\mu}\sigma^2/n)$ on the original scale of measurement (SERFLING 1980). To accommodate for the absence of variance of the zero GUS activity in untransformed, wild-type tobacco, this was given the hypothetical variance of 0.0001, resulting in a high weight in the analysis.

The full regression model would require 49 parameters to describe the seven main effects and all interactions, including reciprocal effects. As expected for biological reasons, the latter were not significant ($P > 0.05$; analyses not shown), indicating that reciprocal crosses

TABLE 2
Geometric means of GUS activity in pooled F₁ populations and segregation of kanamycin resistance in F₂ seedlings

Plant line ^a	NLG-4	NLG-47	NLG-11	NLG-41	ANLGA-13	ANLGA-38	Wild type
NLG-4	17.2 (6) ^b	7.8 (12)	32.0 (12)	38.1 (12)	42.4 (11)	42.5 (12)	22.0 (12)
NLG-47	284:23 ^c	4.2 (6)	10.3 (12)	9.6 (12)	8.8 (12)	9.1 (12)	5.7 (12)
NLG-11	215:12	249:11	609.6 (6)	409.4 (12)	438.4 (12)	423.9 (12)	328.6 (12)
NLG-41	195:13	169:14	374:19	308.2 (5)	214.9 (11)	221.4 (12)	124.4 (12)
ANLGA-13	311:21	881:67	264:15	805:57	211.1 (11)	201.6 (8)	108.9 (12)
ANLGA-38	216:10	157:5	230:9	202:17	815:44	314.7 (6)	132.1 (8)

^a For a description of the plant lines see Table 1. The zero GUS activity of wild type is not included.

^b Values are the geometric mean of GUS activity in picomoles methylumbelliferon per minute per microgram of protein, with the number of plants in the F₁ population in parentheses.

^c Values in boldface are segregation ratios, expressed as number of kanamycin-resistant seedlings: number of kanamycin-sensitive seedlings. The numbers given are the combined results of the evaluated F₂ seedlings obtained from both reciprocal F₁ hybrids. In all cases the segregation follows a 15:1 Mendelian segregation according to the chi-square test for goodness of fit (SOKAL and ROHLF 1995).

could be pooled. One could argue that the reciprocal crosses are the true replicates in this experiment and pooling is not allowed. An analysis using the reciprocal crosses as replicates did not affect the conclusions given below, despite the reduction in statistical power (not shown). For biological reasons, however, we feel that in our experimental setup the replicate plants within crosses are the relevant replicates. The actual geometric means for all pooled 28 combinations are given in Table 2. From the analysis on the logarithmic scale the environmental variance was estimated to be 0.083. This estimate was taken as overall variance σ^2 . The linear regression model was based on 28 parameters, 21 of which describe allele interactions. Of these, six parameters concern dominance, or within-locus interactions, assessed as deviation of the GUS activity in a hemizygous plant from half the GUS activity in the corresponding homozygous plant. The remaining 15 interaction parameters describe epistasis, which was assessed as deviation of the GUS activity in a double hemizygous plant from the sum of the GUS activities of the contributing alleles in hemizygous configuration. Weighted regression analysis showed 11 of the 21 interaction parameters to be highly significant. As a consequence, the full regression model required 18 parameters for an appropriate description of the GUS activities in the 28 F₁ populations of plants. Further analyses showed that all significant dominance and epistasis parameters involved the tobacco lines NLG-4 and NLG-47. The regression analysis partitioned into dominance and epistasis terms for subsets with and without the NLG-4 and NLG-47 alleles, is presented in Table 3.

Additivity of GUS gene activity for one-copy alleles only: For each of the four single-copy lines (NLG-11, NLG-41, ANLGA-13 and ANLGA-38), the parameters for dominance and epistasis are not significant (Table 3). This implies that the homozygous, selfed parents are twice as active as the corresponding hemizygous offspring obtained after crossing to the wild type. More-

over, in all dihybrid combinations of these single-copy alleles, GUS activity is the sum of the activities of the contributing hemizygous alleles. In contrast, all dominance and epistasis parameters involving crosses with the NLG-4 and NLG-47 lines are highly significant, indicating major deviations from simple additivity. Any hybrid in which a one-copy parent is combined with either NLG-4 or NLG-47 has a marked decrease in GUS activity. The GUS activity in such NLG-4- and NLG-47-containing dihybrids is considerably lower than the activity of the one-copy hemizygous offspring obtained after crossing with wild type. Both NLG-4 and NLG-47 impart an epistatically low expression level on all active alleles with which they are combined in this study. In the case of the highest active line, NLG-11, the reduction in GUS activity as result of the combination with NLG-4 is ~20-fold, whereas in combination with NLG-47 the reduction is as much as 60-fold. To exclude that the GUS loci analyzed are closely linked or even identical, genetic linkage was assayed by segregation of kanamycin resistance in F₂ seeds. In all cases the F₂ seeds show a 15:1 segregation for kanamycin resistance (Table 2), demonstrating that the six GUS gene loci are spread over the tobacco genome and assort independently at meiosis. Therefore, in the loci analyzed here, the reduction in GUS activity is apparently independent of the genomic position of the active locus or the presence of MAR elements around the GUS gene.

The two complex loci have low expression and exhibit dominant epistasis and overdominance: The two lines NLG-4 and NLG-47 differ significantly ($P < 0.001$) from each other in GUS activity despite their overall low GUS activity. The difference is approximately fourfold in both the homozygous and the hemizygous configurations. A comparison of the mean of the homozygous NLG-47 plants with the mean of the respective hemizygous NLG-47 × WT plants shows that the hemizygous plants are significantly more active than the homozygous plants. This indicates a within-locus interac-

TABLE 3
Partitioning of mean squares for the ²¹interaction parameters in the 28-parameter model

Pooled parameters	d.f.	MS ^a	F ratio	P ^b
Dominance (within-locus) ^c				
NLG-4 and NLG-47	2	3.600	43.37	***
NLG-11, NLG-41, ANLGA-13 and ANLGA-38	4	0.069	0.83	NS
Epistasis (between-loci) ^d				
All combinations involving NLG-4 and NLG-47	9	7.098	85.52	***
All other combinations	6	0.067	0.81	NS
Residual ^e	254	0.083		

^a Mean square.

^b Probability according to the *F* ratio (SOKAL and ROHLF 1995) relative to the mean square of residuals; NS, not significant ($P > 0.05$); *** $P < 0.001$.

^c Dominance defined as the deviation of the GUS activity of the hemizygous plants from half the GUS activity of the corresponding homozygous plants.

^d Epistasis defined as the deviation of the GUS activity of the double hemizygous plants from the sum of the activities of the contributing alleles in hemizygous configuration.

^e Environmental variance estimated for all 281 natural log-transformed observations, excluding the zero values for the wild type.

tion between the NLG-47 alleles in the homozygous state. The same is true for NLG-4. Therefore, NLG-4 and NLG-47 are examples of plants lines exhibiting classical single-locus overdominance or, depending on the exact definition one prefers, single-locus heterosis ($P < 0.05$).

To interpret the between-loci effects of the NLG-4 and NLG-47 alleles in genetic terms, the segregation of GUS activity of the most extreme combination (NLG-11 × NLG-47) was analyzed in the F₂ after selfing one individual of this F₁ population. In a population of 93 F₂ plants, no evidence for imprinting from either parent was observed. The progeny segregated with 70 low active plants, 18 highly active plants and 5 plants without activity. No evidence for imprinting was observed. This segregation corresponds to the classical 12:3:1 segregation known as dominant epistasis. Therefore, the epistatic interaction imparted by the NLG-47 allele, and presumably also by the NLG-4 allele, is in genetic terms an example of dominant epistasis.

The GUS activity in the F₁ dihybrids containing the GUS allele of NLG-47, excluding the combination with NLG-4, is ~9–10 picomoles methylumbelliferon per minute per microgram of soluble protein, irrespective of the active allele present. This is about fourfold lower than the GUS activity of ~35–40 picomoles methylumbelliferon per minute per microgram of soluble protein in all NLG-4-containing F₁ hybrids, excluding the combination with NLG-47 (Table 2). In both cases, the activity in the dihybrid combination is, however, significantly higher (~1.7-fold; $P < 0.001$) than in monohybrid hemizygous NLG-4 or NLG-47 plants. It is noteworthy that the approximate fourfold difference in activity between dihybrids containing NLG-4 and those containing NLG-47 equals the difference in activity between NLG-4 and NLG-47 plants in either homozygous or hemizygous state.

DISCUSSION

Quantitative analysis: The quantitative behavior of the GUS transgene was assayed in F₁ populations obtained from selfing and intercrossing six transgenic tobacco lines and the wild type. The transgenic lines chosen for analysis differ in the configuration of the T-DNA locus, in the presence or absence of MAR elements flanking the transgenes, and in their relative GUS activities (Table 1). All loci are independently inherited from one another (Table 2). Wild-type tobacco (WT) was included as the corresponding nulliplex plant line, containing no transgene alleles. We constructed a model of the dominance and epistatic interactions using weighted regression analysis of the geometrical mean of GUS activities after pooling the results of reciprocal crosses. Analysis showed that all four single-copy alleles, whether as homozygous or in hemizygous combination with each other, act fully independently, irrespective of the presence or absence of MAR elements around the GUS gene. The non-MAR-contained NLG-11 and NLG-41 alleles cannot be distinguished from the MAR-contained ANLGA-13 and ANLGA-38 alleles.

Reduction of GUS activity is determined by the complex locus: All hybrid combinations of NLG-47 with a single-copy locus (NLG-11, NLG-41, ANLGA-13 or ANLGA-38) are approximately equally active; the same is true for all combinations with NLG-4. The dominant epistatic allele (NLG-4 or NLG-47) determines the level of GUS gene expression in dihybrids, irrespective of the location, molecular configuration (notably MAR presence, see below), or activity of the locus with which it is combined. The fourfold difference in activity between dihybrid offspring containing NLG-4 or NLG-47 may be related to the precise molecular configuration of the NLG-4 and NLG-47 alleles or be determined by the relative position of the epistatically dominant locus

in the interphase nucleus, or be a combination of both. The GUS activity in dihybrid combinations containing NLG-4 or NLG-47 is ~1.7-fold higher than the activity of the NLG-4 or NLG-47 allele in the hemizygous configuration. This most likely indicates that the active locus is not repressed completely. At the moment, however, it cannot be excluded that the NLG-4 and NLG-47 alleles are epistatically stimulated in dihybrid combinations.

The two dominant epistatic alleles NLG-4 and NLG-47 also show single-locus overdominance: hemizygous plants are more active than the corresponding homozygous plants. A comparison of the NLG-4 and NLG-47 allele-carrying plants shows an approximately fourfold difference in GUS activity, both when hemizygous, homozygous or dihybrid plants are compared. This indicates that the phenomena of epistatic dominance and overdominance are equally and quantitatively affected by the presence of either NLG-4 or NLG-47. Therefore, the epistatic dominance, resulting in between-loci reduction of GUS activity, is likely to be based on the same molecular mechanism as the single-locus overdominance that results in a within-locus reduction of GUS activity.

The epistatic reduction in GUS activity is not overcome by flanking MAR elements: Any new transgene or transgene-associated sequence may confer or be subject to epistatic gene interaction. As little as 90 bp of promoter homology can be sufficient for efficient gene silencing (VAUCHERET 1993). As long as the mechanisms of epistatic gene silencing are not resolved, or molecular safeguards are developed, progeny testing is required. The MAR sequences evaluated here could have been such safeguards. It has frequently been suggested that MAR sequences may protect against (some) silencing phenomena (FINNEGAN and McELROY 1994; FLAVELL 1994; MEYER 1995a). The results shown here demonstrate that the MAR elements around the transgene do not protect against the NLG-4- and NLG-47-mediated reduction in GUS activity. The activity of the MAR-carrying ANLGA-13 and ANLGA-38 alleles is reduced as much as the non-MAR NLG-11 and NLG-41 alleles. This result could relate to the particular MAR element used in our studies, the chicken lysozyme A element. However, the significant influence of this element on transgene expression in plants previously observed (MLYNÁROVÁ *et al.* 1994, 1995), as well as its affinity for the nuclear matrix of tobacco (MLYNÁROVÁ *et al.* 1994), suggest that the A element is an active MAR in plants. Such MARs are supposed to create a chromatin domain that is transcriptionally less dependent on the surrounding DNA.

In terms of the various gene silencing phenomena known to date (MATZKE and MATZKE 1993, 1995; FINNEGAN and McELROY 1994; FLAVELL 1994; PASKOWSKI 1994; MEYER 1995b), the NLG-4 and NLG-47-mediated reduction in GUS activity is an example of homology-

dependent gene silencing in which the combination of two similar or identical genes induces the silencing of gene expression. Similar reductions in GUS activity in tobacco have been observed by HOBBS *et al.* (1990, 1993). The mechanism of this type of homology-dependent silencing is not understood yet, but evidence is accumulating that the mechanism requires homology of coding sequences and complex T-DNA loci (HOBBS *et al.* 1990, 1993; ASSAAD *et al.* 1993) and is in some way triggered post-transcriptionally, either by ectopic interactions or aberrant RNA formation (BAULCOMBE and ENGLISH 1996). The results shown here indicate that it is not sufficient to distinguish between "high" and "low" expressing plants as defined by HOBBS *et al.* (1993). Both the NLG-4 and NLG-47 lines should be classified as low, but they clearly differ from each other. This difference may be related to the position in the genome or to the precise configuration of the transgenes in NLG-4 compared with NLG-47. The observation that the presence of MARs around the T-DNA does not protect against this type of homology-dependent reduction in gene activity supports the notion that it is a posttranscriptionally regulated phenomenon. Our analyses of the dominant epistasis and overdominance exhibited by the NLG-4 and NLG-47 alleles would seem to favor a quantitative mechanism underlying homology-dependent gene silencing. More detailed analyses of the precise characteristics of the NLG-4 and NLG-47 alleles will be required to obtain more insight into this mechanism.

Interfacing quantitative with molecular genetics: Current developments in gene mapping, marker-assisted selection and QTL mapping (TANKSLEY 1993; DEAN and SCHMIDT 1995; LAW 1995; JANSEN 1996) show that plant molecular biology and quantitative genetics have become intimately connected in the area of gene localization.

Recently it was pointed out that putative epistatic interactions complicate QTL mapping and the genetic interpretation of mapping results considerably (FIJNEMAN *et al.* 1996; FRANKEL and SCHORK 1996). The putative interactions between different QTLs will result in so rapid an increase in the number of parameters required that the amount of data available will quickly become limiting. Therefore, the detection and quantification of epistatic interactions will require a different experimental approach. Analyses as outlined in this paper show how complex traits could be studied by further combining the tools of molecular and quantitative genetics. GUS activity in the transgenic setup chosen here can be considered a synthesis of a simple, two-locus quantitative trait. Tobacco is an inbreeding amphidiploid species (WERNSMAN and MATZINGER 1980). Therefore, the seven tobacco lines analyzed are fully isogenic except for the T-DNA, apart from putative disturbances caused by tissue culture steps or the T-DNA insertion. This allows a powerful analysis of gene action and interac-

tion, undisturbed by differences in genomic background. The data presented show that different combinations of single-locus transgenic loci may yield simple quantitative traits in which varying contributions of between-loci and within-locus interactions can occur and can be analyzed. Constructing a quantitative trait from single, well-characterized loci and analyzing the results with the tools of quantitative genetics can prove useful in further dissecting complex traits in terms of gene action and gene interaction. This approach is likely to contribute to our understanding of the performance of (trans)genes in complex traits in plants.

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