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Combined genetic and physiological analysis of a locus contributing to quantitative variation

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Abstract The natural variation of many traits is controlled by multiple genes, individually referred to as quantitative trait loci (QTL), that interact with the environment to determine the ultimate phenotype of any individual. A QTL has yet to be described molecularly, in part because strategies to systematically identify them are underdeveloped and because the subtle nature of QTLs prevents the application of standard methods of gene identification. Therefore, it will be necessary to develop a systematic approach(es) for the identification of QTLs based upon the numerous positional data now being accumulated through molecular marker analyses. We have characterized a QTL by the following three-step approach: (1) identification of a QTL in complex populations, (2) isolation and genetic mapping of this QTL in near-isogenic lines, and (3) identification of a candidate gene using map position and physiological criteria. Using this approach we have characterized a plant height QTL in maize that maps to chromosome 9 near the centromere. Both map position and physiological criteria suggest the gibberillin biosynthesis gene *dwarf3* as a candidate gene for this QTL.

Key words Quantitative trait locus (QTL) · Maize · Molecular markers · *dwarf3* · Gibberellin · Plant height

Introduction

Many traits of agricultural, biological, and medical significance exhibit "quantitative" inheritance (Falconer 1981; Strickberger 1968), in that multiple genes act in concert with environmental factors to determine the natural variation observed for that particular trait. Because these genetic effects are assumed to be complex and not generally resolvable into individual factors, quantitative geneticists have largely dealt with their characterization en masse using biometrical approaches. Consequently very little is known about the biological nature of quantitative or "natural" variation in terms of individual genes and their biochemical functions.

Sax (1923) first suggested examining the linkage of quantitative variation to marker genotypes to facilitate the analysis of quantitative genes. This strategy was largely impractical until the later application of isozymes and restriction fragment length polymorphisms (RFLPs) to develop saturated genetic maps for many organisms. Recent studies in both plants (Helentjaris, unpublished; Helentjaris and Shattuck-Eidens 1987; Nienhuis et al. 1987; Peterson et al. 1988; Stuber et al. 1992) and animals (Jacob et al. 1991; Anderson et al. 1994) have demonstrated that a limited number of chromosomal regions, often referred to as quantitative trait loci (QTLs), can be defined that control a significant portion of the phenotypic variation for any trait. Another strategy with this general aim, the "candidate gene" approach, attempts to correlate variation in a quantitative trait with a candidate gene in a related biochemical or physiological process (Winkelman and Hodgetts 1992; Gray and Tait 1993). Although confirmation of the relationship of specific QTLs and candidate genes has proven difficult, in some instances the genomic location of the candidate gene has been examined in a population segregating for both variation in the trait and "alleles" of the candidate gene, providing further support for the relationship. While this approach has been applied in animal systems, its resolution is often limited due to the complexity of the populations, the frequent lack of polymorphism associated with individual candidate gene probes, and the limited number of linked marker loci available. A third approach suggested by Donald Robertson (1985) is that quantitative variation and qualitative variation might often represent the actions of alternative alleles at the same loci and

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that this provides the means for identification and cloning of individual genes involved in quantitative variation through the initial isolation of those alleles with more extreme effects upon phenotype. Several researchers have since noted that molecular marker analysis does indeed locate some major quantitative factors nearby to mutant alleles with related but more extreme effects upon the organism's phenotype (Beavis et al. 1991; Edwards et al. 1992; Helentjaris, unpublished; Helentjaris and Shattuck-Eidens 1987), supporting Robertson's contention. Despite these advances in both our knowledge of, and strategies for studying natural variation, a QTL as defined by these types of approaches has yet to be molecularly identified.

We believe that a combination of these strategies provides the basis for a realistic approach for identifying, cloning, and studying such genes. In this approach the first step is to utilize molecular marker scanning of the effects within segregating populations of individual regions covering the entire genome to determine the locations of major QTLs. These regions are each evaluated for either candidate genes or mutants with similar but more extreme effects upon phenotype. We have subsequently incorporated the use of near-isogenic lines (NILs) to obtain a better measure of their actual impact on phenotypic variation as well as a more precise localization of the target QTL(s). NILs eliminate the confounding effects of all other genomic regions, a useful attribute as long as there are no epistatic interactions between the target QTL and any other genomic regions. Lastly, we have employed a physiological analysis to provide a confirmation of our candidate gene/mutant that is independent of positional information.

As a model trait for evaluation of this strategy, we have initially chosen plant height in maize, which is known to be quantitatively inherited and where a search for major QTLs using segregating progenies has previously revealed correspondence in some cases to the map positions of dwarf mutants (Beavis et al. 1991; Edwards et al. 1992; Helentjaris, unpublished; Helentjaris and Shattuck-Eidens 1987). In particular, a region around the centromere of chromosome 9 (9c) exhibits a strong effect upon height in several genetic backgrounds and also contains the candidate loci, *d3* (*dwarf3*), a gibberellin (GA) biosynthetic gene (Fujioka et al. 1988) whose mutant forms exhibit a strong effect upon height, and *phyB*, a phytochrome gene involved in light level recognition whose over-expression can result in dwarfed plant phenotypes very similar to those produced by GA mutants (Boylan and Quail 1991). Our goal in this study was to begin to develop and test a general approach to isolating individual genes acting as QTLs using the type of positional information initially gained from molecular marker analyses as a starting point.

Materials and methods

Plant material

The maize chromosome 9c region contains the endosperm-expressed gene *waxy* for which numerous NILs have been produced by using a donor *waxy* inbred that is normally backcrossed at least 8 times to the recipient normal line. The final line is derived by pooling multiple-sibling backcrosses and therefore would be expected to contain individuals that are nearly identical, but which might also vary in the exact length of the introgressed segment and possibly in some regions that are not linked to the introgressed region. After eight backcrosses these NILs would result in a theoretical introgression of 20–30 cM. The *d3* gene has been genetically mapped to within 3 cM of the *waxy* gene, and so it is likely to have been simultaneously introgressed along with *waxy* in these efforts. Maize *phyB* has been mapped to 28 cM distal to *waxy* on the long arm and might be expected to be included with some introgressions. Over 60 NIL pairs for this genomic region were collected for study, each consisting of an original recurrent line and its *waxy* conversion line.

Plant growth and phenotypic analysis

Plants were grown either in individual 2-gallon pots in a greenhouse with lighting supplemented to 16-h days or during the 1993 summer field season in Tucson, Arizona, as space-planted individuals with drip irrigation. Height measurements were taken at various points during the growing season as to the highest point of the growing plant. To define levels of GA that are physiologically relevant, we analyzed the growth response of *d3-Ref* mutant plants to varying levels of GA_3. The GA-deficient dwarf is sensitive to GA addition and thus provides a criterion for analyzing the GA sensitivity of the original and converted lines. We assumed that GA levels that elicit a significant growth response for GA-synthesis deficient *d3-Ref* plants but yet are insufficient to normalize the growth of *d3-Ref* plants relative to the wildtype should be physiologically relevant to the normal variation in plant height.

Southern analysis of plants

Genomic DNA preparation was by standard procedures (Helentjaris et al. 1986; Chen and Dellaporta 1994). All Southern blot procedures were done by a non-radioactive procedure using probes labeled with digoxigenin-dUTP (Boehringer Mannheim). Most probes for Southern analysis were labeled by polymerase chain reaction (PCR) using 5% digoxigenin-dUTP, but longer probes (>1.5 kb) were prepared by oligo-labeling (Genius kit, Boehringer Mannheim). Hybridizations were done at 65°C in 0.25 *M* Na_2HPO_4 buffer, pH 7.4, 7% SDS, 1% gelatin, 1.0 m*M* EDTA. After a 30-min wash at 65°C in 0.15×SSC and 0.1% SDS, blots were processed by the procedures outlined in Boehringer Mannheim Catalogue Number #101023 V. 2.0 with minor modifications. AMPPD or CSPD (Tropix) were used as alkaline phosphatase substrates.

Results

Genotypic analysis of NILs

Prior work had already pinpointed a genomic region, near the centromere of chromosome 9, which was detected in many different genetic backgrounds as a major QTL for height (Helentjaris, unpublished) and which contained the candidate genes, *dwarf3* (*d3*) and *phytochromeB* (*phyB*). We analyzed over 60 NIL pairs with RFLP probes that detect chromosome 9 loci by Southern analysis (one example is illustrated in Fig. 1). These data suggested that all NILs had the same *waxy* allele introgressed into these different inbred backgrounds. Given the high rate of polymorphism detected by this particular probe, it can be seen that a variety of alternative *waxy* genes (and by inference the

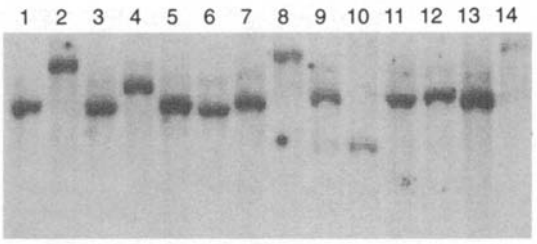

Fig. 1 Genotyping of NIL pairs for chromosome 9c. Genomic DNA from seven pairs of 9c NILs were digested with *Eco*RI and analyzed by Southern analysis with a *waxy* probe. Paired lanes (*1–2, 3–4, 5–6, 7–8, 9–10, 11–12, 13–14*) in each case represent a different *waxy* introgression line (*odd lanes*) with its original recipient line (*even lanes*). The *waxy* fragment (*odds*) in each case is identical in molecular weight across all introgressions

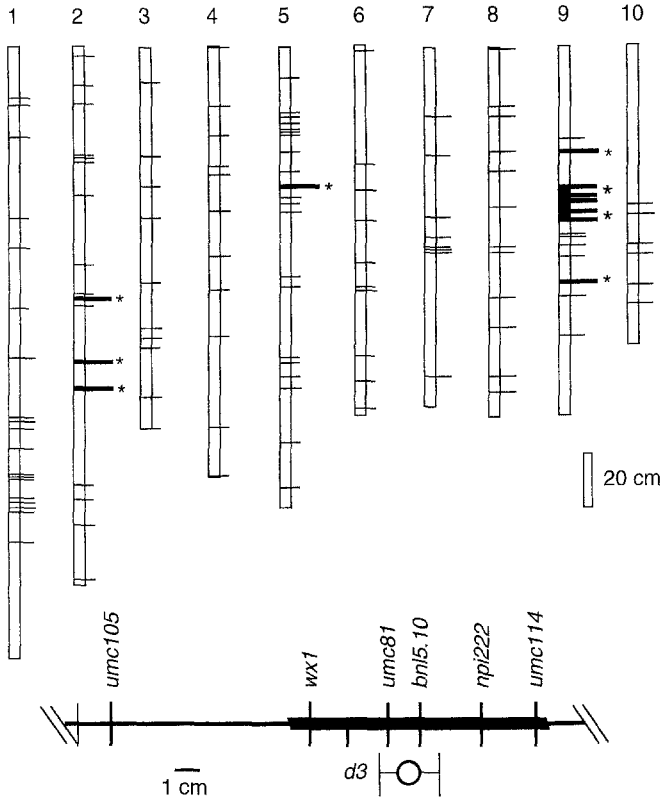

Fig. 2 Genotyping of the W153R NIL pair. DNA was prepared from pools of plants from the W+ and W9c inbreds and analyzed by Southern blotting with probes from around the genome using three restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII for genomic digestion. The maize chromosomes are each diagramatically represented by a *vertical bar* on which the genotyped loci are denoted by a *horizontal tic*, indicating their relative map locations. Those loci detected as polymorphic between the W+ and W9c versions are marked with *bold horizontal tics* and an *. *Below*, the region of chromosome 9 which contains those markers polymorphic between W+ and W9c and including *d3* is illustrated

genes linked to them) were replaced by this single 9c cassette. RFLP analysis of these NILs showed that the size of this introgressed region was much larger than the *waxy* gene alone and did approximate the predicted size of at least 20–30 cM (data not shown). Consequently, one might expect that for a number of these NIL pairs, a quantitative difference for height might be detected due to the replacement of alleles at any major QTLs found in the introgressed region.

Among the numerous NIL pairs that were analyzed for height, one pair based upon the inbred W153R, which was one of the shorter inbreds tested among this set, exhibited a significant difference between the normal inbred (referred to as W+) and its 9c conversion (referred to as W9c). Other pairs revealed significant but smaller phenotypic differences. To verify that the phenotypic differences we observed could be ascribed to the 9c region and that this introgression also included the candidate genes, we genotyped the W153R NIL pair with molecular markers not only from 9c but also from across the maize genome. As is illustrated in Fig. 2, Southern blot analysis of W+ and W9c using RFLPs defined an introgressed segment of approximately 12 cM on chromosome 9 (markers *wx* to *umc114*), a region expected to contain the *d3* gene but not the *phyB* gene (Matz et al. 1994). The RFLP marker *umc105*, which is distal to *wx* on 9S, was not fixed in the W+ inbred; two alleles were present in the W+ inbred population. The W9c line was fixed for one of these two alleles. Since *umc105* is the closest marker to *wx* on chromosome 9S, it is not possible to know if this segment was also introgressed with the mutant *wx* allele, but given the highly polymorphic nature of maize it may not necessarily represent a contiguous introgressed fragment. Additional Southern blot analysis also defined a 40-cM region on chromosome 2L (markers *csu4* to *umc5*) in some sibling members of this *waxy* NIL, along with two other introgressed regions defined by only a single marker: chromosome 9L (defined by *npi403*, 50 cM distal to *d3* on the long arm) and chromosome 5S (defined by *npi213*). Some individuals (<5%) in the W9c population had smaller introgressed fragments at 9c, and other individuals (<5%) did not contain the other regions, which is not surprising given the nature as to how these NILs are produced. Given this complexity of introgression, one could not be certain how much, if any, of the phenotypic variation for height between the W+ and W9c versions was in fact due to the 9c region itself.

Phenotypic analysis of the W153R NIL pair

To evaluate the contribution of individual regions for plant height in this NIL, we analyzed the progeny of a self of W+/W9c heterozygotes, which should only be segregating for the regions described above, both phenotypically and with molecular markers. Plants were grown in the greenhouse (n=111) and analyzed for height at 3-weeks post-emergence. Plant material was also collected, genomic DNA prepared, and Southern analysis performed using those markers detected as segregating in the original anal-

Table 1 Effect of allelic substitution at segregating loci upon the phenotypic variation for height

Genomic regulation[a]	Geno-type[b]	Mean height	SD	*F* ratio	*P*>F[c]	R^{2d}
2L	0	86.4	8.3	2.79	0.0656	0.05
	1	87.9	8.4			
	2	83.5	5.6			
5s	0	88.3	6.7	1.43	0.234	
	1	86.1	8.3			
9c	0	82.1	8.6	7.17	0.0012	0.12
	1	88.5	7.1			
	2	86.1	7.2			

[a] Only those regions exhibiting polymorphism between the two parental lines are reported. The chromosomal region 2L was detected by *umc55* (n=111), chromosome 5S was evaluated with *npi213* (n=109), and chromosome 9c was evaluated with *umc114* (n=111)
[b] Genotypic classes are coded as 0 for the homozygous W+ genotype, 1 for the heterozygous genotype, and 2 for the homozygous W9c genotype
[c] Significance level of the *F* ratio
[d] Region's contribution to the total variance

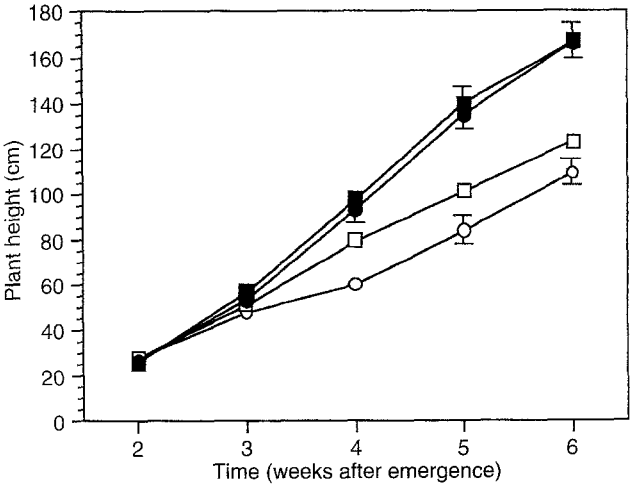

Fig. 3 Height measurements of W+ and W9c pairs and the effect of exogenous GA addition. Plants were grown in the greenhouse and measured for height each week beginning 2 weeks after emergence. Height values represent the means of seven to eight plants with error bars equal to one-half of the least significant difference at probability level 0.05. Means are significantly different where error bars do not overlap. *Circles* represent original inbred (W+), and *squares* represent the converted version (W9c). *Open symbols* represent control-untreated plants, and *filled symbols* represent those treated with GA_3

ysis. Using both the genotypic and phenotypic data, we performed a one-factor analysis of variance with PROC ANOVA, (SAS Institute 1985) to detect the effect of individual regions upon height (Table 1). A highly significant effect was observed for the 9c region containing *d3* (P=0.0012) while, conversely, the chromosome 2L and 5S regions did not exhibit a significant effect upon plant height. In parallel field experiments, plants resulting from either self-pollinations of several W9c individuals, backcrosses to the W+ version, and from self-pollinations of W+/W9c heterozygotes were analyzed for mature plant height (n=89) and subsequently by Southern hybridization with markers from those regions previously defined as polymorphic between W+ and W9c. Again a significant effect was detected with the 9c region at a level of .0001 and the effects of the other regions were much less significant (data not shown). So although other genomic regions were found to be segregating within this particular NIL conversion, these regions exhibited little effect upon the variation for plant height observed between these two highly-related lines. Through both field and greenhouse testing, most of the phenotypic variance for height between the normal and converted versions of this line can be ascribed to genes contained within the 9c region, confirming earlier results from populations segregating for the entire genome that originally demonstrated a major QTL near 9c.

Identification of *dwarf3* as a candidate gene

If the basis of the height QTL in the 9c region is allelic variation at the *d3* locus, then we should be able to make further predictions about the nature of their effect upon this trait. In particular, as *d3* is involved in the biosynthesis of GA, then the primary effect of different alleles at this locus upon height should be due to varying levels of GA, as is true in available mutants. By artificially altering these levels in two related inbreds differing for 9c, through exogenous application of GA, we should be capable of eliminating the phenotypic differences between these two lines. The results of one such test are shown in Fig. 3. As observed previously, the untreated W9c NIL was significantly taller than the untreated W+ inbred over the entire growth period in greenhouse conditions. Closer comparison of these individuals revealed that this difference between original and converted lines was primarily due to a change in internode length and not in the number of nodes, an outcome that is also associated with GA-biosynthetic mutants (Coe et al. 1988). The addition of GA resulted in a growth response in both normal and converted versions, but more importantly it also removed any measurable difference in the height of the normal and converted versions when they were directly compared at any point in their growth. This effect of exogenously added GA to eliminate the height difference between the two versions was evident at several different concentrations (data not shown). GA levels have been proposed to be a limiting factor for plant growth (Reid and Ross 1993). These studies in combination with mapping data from segregating families (Beavis et al. 1991; Edwards et al. 1992; Helentjaris et al. unpublished; Helentjaris and Shattuck-Eidens 1987) make the GA-biosynthesis gene *d3* an excellent candidate QTL. Our data using phenotypic comparisons of NIL, genetic mapping of QTL, and physiological criteria give critical support to this hypothesis. That the difference in height

between both NIL pairs is compensated by external addition of GA indicates that GA levels are a limiting factor in plant growth for these genotypes under these conditions. This difference also must eventually rest upon a genetic explanation, implicating the allelic variation of genes involved in the pathways of biosynthesis, response and regulation for this plant hormone. Since this difference in height is primarily attributed to a difference in the 9c region, a relatively small chromosome region containing a gene involved in the biosynthesis of GA, then *d3* must be considered to be a strong candidate for this QTL.

Molecular analysis of *dwarf3* and *phytochromeB* as candidate genes

We have begun follow-up studies to confirm and study the possible involvement of the *d3* and *phyB* genes in this quantitative effect. Using clones for *d3* (Winkler and Helentjaris, submitted) and a rice *phyB* (provided by P. Quail) as hybridization probes, we analyzed the two W153R lines by Southern analysis. The *d3* probe revealed several restriction site polymorphisms between the normal and converted lines (*Bam*HI, *Eco*RI, and *Xba*I) consistent with the fact that the surrounding loci, *wx*, *umc81*, *bnl5.10*, *npi222*, and *umc114*, are also polymorphic. On the other hand, the *phyB* probe reveals no such RFLPs, as might be expected from its presumed genomic location outside the actual introgressed region in this pair (data not shown). Allelic variation at *d3* in combination with the QTL map position and physiological criteria suggest that a *d3* allele has been introduced into W9c, which may be responsible for the quantitative variation associated with this region. Given this result, it would now seem appropriate to evaluate the various resident *d3* genes in the original recipient lines as compared to that in the introgressed cassette for differences in gene expression and to attempt to correlate those differences with the phenotypic variation observed between the recipient and converted lines.

Discussion

This study reports on the identification of a candidate gene for "natural" or quantitative variation by a combined genetic and physiological approach. In this approach we have utilized previous data obtained from genomic scanning by molecular markers to prioritize amongst possible candidate genes/mutants, to verify NILs, and more precisely to measure the actual effect of genes in that region, and subsequent physiological evaluations to confirm the identity of the presumed candidate gene. This type of approach not only provides support for but also significantly improves upon Robertson's original hypothesis as a general strategy for identifying such genes. The natural extension of this general strategy is to now use clones for these candidate QTL genes to answer some basic questions about individual gene contributions to quantitative variation by assaying for allelic differences in gene expression and how these differences do indeed correlate with the phenotypic variation of a quantitative nature. These types of studies would then provide biological data to accompany prior statistical insights into the nature of genes affecting natural variation. If the results and strategy of this study prove generally applicable to other traits and species, then the powerful tools of molecular genetics and genetic engineering reserved in the past for "Mendelian" genes will now also become available for the improvement of complex characters in both plants and animals.

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