Unravelling epistasis with triple testcross progenies of near isogenic lines

Jochen C. Reif*,†, Barbara Kusterer*, Hans-Peter Piepho‡, Rhonda C. Meyer§, Thomas Altmann§, Chris C. Schön** and Albrecht E. Melchinger*,†

* Institute of Plant Breeding, Seed Science, and Population Genetics, University of Hohenheim, 70599 Stuttgart, Germany
† State Plant Breeding Institute, University of Hohenheim, 70599 Stuttgart, Germany
‡ Institute of Crop Production and Grassland Research, Bioinformatics Unit, University of Hohenheim, 70599 Stuttgart, Germany
§ Department of Molecular Genetics, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany
** Institute of Plant Breeding, Technical University of Munich, 85350 Freising, Germany
Running head: Mapping epistatic QTL

Keywords: Epistasis, near-isogenic lines, triple testcross design, Arabidopsis, QTL

1 Corresponding author: Institute of Plant Breeding, Seed Science, and Population Genetics, University of Hohenheim, Fruwirthstr. 21, 70599 Stuttgart, Germany. Phone: ++49-711-459-22334, Fax: ++49-711-459-22343, E-mail: melchinger@uni-hohenheim.de
ABSTRACT

Libraries of near-isogenic lines (NILs) are a powerful plant genetic resource to map quantitative trait loci (QTL). Nevertheless, QTL mapping with NILs is mostly restricted to genetic main effects. Here we propose a two-step procedure to map additive x additive digenic epistasis with NILs. In the first step, a generation means analysis of parents, their F₁ hybrid, as well as one-segment NILs and their triple testcross (TTC) progenies is used to identify in a one-dimensional scan loci exhibiting QTL by background interactions. In a second step, one-segment NILs with significant additive x additive background interactions are used to produce particular two-segment NILs to test for digenic epistatic interactions between these segments. We evaluated our approach by analyzing a random subset of a genome-wide Arabidopsis thaliana NIL library for growth-related traits. The results of our experimental study illustrated the potential of the presented two-step procedure to map additive x additive digenic epistasis with NILs. Furthermore, our findings suggested that additive main effects as well as additive x additive digenic epistasis strongly influence the genetic architecture underlying growth-related traits of Arabidopsis thaliana.
Quantitative traits are affected by many genes that act singly and in interaction with each other. Epistasis, the interaction between genes at different loci, may exert important effects on (1) the dynamic of evolving populations (CHEVERUD and ROUTMAN 1996), (2) changes of genetic variances caused by long-term selection (CARLBORG et al. 2006) or by a population bottleneck (GOODNIGHT 1987), and (3) heterosis (DOEBLEY et al. 1995; YU et al. 1997; LI et al. 2001; XING et al. 2002; HUA et al. 2003, MEI et al. 2003, SYED and CHEN 2005; KUSTERER et al. 2007; MELCHINGER et al. 2007a).

To detect epistatic QTL in conventional mapping studies with segregating populations such as recombinant inbred lines (RILs), methods have been applied to search multiple QTL simultaneously (for review see CARLBORG and HALEY 2004). Such multidimensional scans are hampered by the problem of multiple tests, which increases for digenic epistasis in a quadratic manner compared to tests for presence of main effect QTL. Consequently, extremely high critical thresholds must be applied for each individual test to warrant a given genome-wide type I error rate. This results in drastically reduced power to detect significant QTL interactions (LANDER and BOTSTEIN 1989).

The problem of multiple tests becomes even more severe when investigating higher-order epistasis.

Several approaches have been applied to tackle the problems related to multiple testing. Searches for epistatic interactions have been limited to certain portions of the genome, e.g., considering only QTL regions with significant main effects (FIJNEMAN et al. 1996). Nevertheless, epistatic QTL with no strong main effects will then not be detected (HOLLAND et al. 1997). Alternatively, JANNINK and JANSEN (2001) proposed the use of multiple related inbred line crosses for detecting epistasis. In their ap-
proach, epistatic QTL are mapped by identifying loci with significant interactions be-
 tween QTL and genetic background in a one-dimensional genome-scan by combining
 information from the different crosses. Alternatively, multiple QTL mapping has been
 proposed with various model selection methods from both frequentist and Bayesian
 perspectives (for review see YI et al. 2007). Extending multiple QTL mapping
 (JANSEN 1993; JANSEN and STAM 1994), BOER et al. (2002) presented a one-
dimensional genome scan for detecting interacting QTL in a single population. Their
 approach uses penalized likelihood methods to fit the interaction between a QTL and its
 genetic background.

 Libraries of near-isogenic lines (NILs) are a powerful plant genetic resource to
 map main effect QTL (LYNCH and WALSH 1998) and QTL by background interac-
 tions (MELCHINGER et al. 2007a). In addition, two-segment NILs produced from pa-
 rental one-segment NILs crossed in a diallel mating design facilitate the detection of
digenic epistatic effects (cf. ESHED and ZAMIR 1996). It is very cost- and time-
 intensive, however, to establish and phenotype a genome-wide two-segment NIL li-
brary. For instance, using the genome-wide Arabidopsis NIL library with Col-0 back-
ground established by TÖRJÉK et al. (2008), which consists of 78 one-segment NILs,
would result in 3003 possible two-segment NILs. Hitherto, selection of parental one-
segment NILs has been based on QTL main effects (ESHED and ZAMIR 1996), but
this selection criterion is problematic, because the presence of a QTL main effect does
not necessarily point to the presence of epistatic effects (HOLLAND et al. 1997).

 The genetic basis of heterosis was investigated by applying the TTC design
 (KEARSEY and JINKS 1968) for RIL (KUSTERER et al. 2007) and NIL populations
 (MELCHINGER et al. 2007a) in Arabidopsis. The power of QTL detection of genetic
main effects was higher with NILs than RILs taking into account the size of the mapping population (MELCHINGER et al. 2007a). Digenic epistatic effects were estimated for RIL populations using linear transformations from genetic values of triple testcross (TTC) progenies (KUSTERER et al. 2007), but for the NIL population, detection for epistasis was restricted to QTL by background interactions (MELCHINGER et al. 2007a). For QTL by background interactions, NIL also showed an advantage compared to RIL populations (MELCHINGER et al. 2007a). It is therefore of interest to investigate the potential of NIL populations for estimating particular digenic epistatic effects.

Here we propose a two-step procedure to efficiently map additive x additive digenic epistatic effects within NIL populations extending the approach of MELCHINGER et al. (2007a). The first step consists in the application of a generation means analysis of inbred parents and their F₁ hybrid, as well as one-segment NILs and their TTC progenies to identify loci exhibiting QTL by background interactions in a one-dimensional scan. In a second step, one-segment NILs with significant additive x additive background interactions are used to produce particular two-segment NILs to test for additive x additive digenic epistatic interactions between these segments. We evaluated the potential of our approach by analyzing a random subset of the genome-wide Arabidopsis thaliana NIL library established by TÖRJÉK et al. (2008) for growth-related traits. The QTL detection power for epistatic effects of the NIL population was then compared with that of an already published study based on a RIL population established from the same cross (KUSTERER et al. 2007).
THEORY

Assume two parents P1 and P2, which differ at the loci set $Q = \{1, ..., q\}$. Following MELCHINGER et al. (2007b), the genotypic value of a genotype $V = (v_1, ..., v_q)$ can be expressed using the F2-metric (COCKERHAM 1954; YANG 2004) and a genetic model including additive x additive epistasis,

$$ G_V = \mu + \sum_{i \in Q} r_i a_i + \sum_{i \in Q} u_i d_i + \sum_{\{i, j\} \in Q} t_{ij} a_{ij}, \quad [1] $$

where $\mu$ = mean of the F2 generation in linkage equilibrium produced from the cross of parents P1 and P2,

$a_i$ = additive effect of locus i (which is positive or negative depending on whether parent P2 or P1, respectively, carries the favourable allele at this locus),

$d_i$ = dominance effect of locus i,

$aa_{ij}$ = additive x additive effect between loci i and j,

$v_i$ = 0, 1, or 2 if the genotype at QTL i is homozygous P1, heterozygous or homozygous P2, respectively,

$$ r_i = v_i - 1, \quad u_i = 2v_i - v_i^2 - \frac{1}{2}, \quad t_{ij} = (v_i - 1)(v_j - 1). $$

If we define the parameters $[a] = \sum_{i \in Q} a_i$, $[d] = \sum_{i \in Q} d_i$, $[aa] = \sum_{\{i, j\} \in Q} a_{ij}$, $[aai] = \sum_{j \in Q \setminus i} a_{aij}$, and denote a possible cytoplasmic effect attributable to seed parent P1 versus seed parent P2 by $c$, then we can express the generation means of (1) parents P1 and P2, their F1 cross, (2) NIL1-i of P1 with one genomic region harbouring locus i from parent P2 in the genetic background of parent P1, (3) NIL1-ij of P1 with two genomic regions, one harbouring locus i and the other harbouring locus j from parent P2 in the genetic background of parent P1, (4) NIL2-i or NIL2-ij being analogously defined but harbouring one or two genomic regions from parent P1 in the genetic background of parent P2, and (5) TTC progenies of each NIL1-i, NIL1-j, NIL1-ij or NIL2-i, NIL2-j, NIL2-ij (i.e., crosses with P1, P2, and F1) as follows:
\[ G = \mu + w + x [a] + y [d] + z [a] + x_i a_i + y_i d_i + z_i [aa_i] + x_j a_j + y_j d_j + z_j [aa_j] + z_{ij} aa_{ij} \]  

with coefficients \( u, x, y, z, x_i, y_i, z_i, x_j, y_j, z_j \) and \( z_{ij} \) as given in Supplementary Table S1. Equation 2 is an extension of the model presented by MELCHINGER et al. (2007a) adding an effect for the (1) second introgressed segment and (2) interaction between both introgressed segments.
MATERIAL AND METHODS

**Plant materials:** We used a subset of the NIL library established by TÖRJÉK et al. (2008) (Supplementary Figure S1, Supplementary Table S2). Briefly, our study comprised ten NILs with one chromosome segment from parent Col-0 in the genetic background of parent C24, denoted as NIL1-s (s = 1,2,…,10). In addition, five NILs were used with one chromosome segment from parent C24 in the genetic background of parent Col-0, denoted as NIL2-s (s = 1,2,…,5).

By crossing pairs of one-segment NILs, NILs were derived harbouring exactly two introgressed segments, denoted as NIL1-st or NIL2-st. Parents of the two-segment NILs were selected randomly. Our study comprised 14 NIL1-st (st = (1,3)-a, (1,3)-b, (1,4), (3,2)-a, (3,2)-b, (5,6), (7,10)-a, (7,10)-b, (7,10)-c, (9,1), (9,2), (9,3), (9,7), (9,8)) with the genetic background of parent C24. Moreover, five NIL2-st were used (st = (2,5), (3,1), (3,4)-a, (3,4)-b, (3,5)) with the genetic background of parent Col-0. Some combinations of two-segment NILs were produced repeatedly (indicated by “-a”, “-b”, and “-c”), where the replications varied slightly with regard to the length of the introgressed segments. Based on these replications, the influence of small differences in the introgression length on the estimated genetic effects were determined.

To facilitate production of testcross seed, we established near-isogenic male-sterile lines of C24 and Col-0, subsequently referred to as P1 and P2, respectively (for a detailed description see KUSTERER et al. 2007). The F₁ generation between P1 and P2 was produced in both reciprocal forms: P1 × P2 (F₁-a) and P2 × P1 (F₁-b). Moreover, testcross progenies were produced according to a TTC design (KEARSEY and JINKS 1968) by mating each NIL1-s and NIL1-st as pollen parent with P1, P2, and F₁-a, as well as each NIL2-s and NIL2-st as pollen parent with P1, P2, and F₁-b. One representative plant of each NIL was used to pollinate three plants of each tester (P1, P2, F₁-a, or F₁-b) and was self-pollinated for seed increase of the
NIL. In all instances, apart from six siliques per mother plant, all others were removed to warrant a homogeneous seed size for minimizing maternal effects.
Greenhouse trials: The 140 genotypes (34 NILs, their 102 TTC progenies, P1, P2, F1-a, and F1-b) were evaluated in a split-plot design with three replicates. Main plots were arranged in an α-design. Each main plot comprised four entries: (1) one NIL and its three testcross progenies produced by the TTC design or (2) P1, P2, F1-a and F1-b (included twelve-times as entries at the main plot level). In all instances, the entries within each main plot were randomly assigned to the subplots. Each sub-plot consisted of 10 plants per entry.

Growth conditions and trait measurements were described in detail by KUSTERER et al. (2007). Briefly, each individual plant was evaluated for rosette diameter (in millimetres) at 22 days (RD22) and 29 days after sowing (RD29), leaf area at 22 days (LA22) and 29 days after sowing (LA29), absolute growth rate per day (GR; in millimetres per day), dry matter content (DMC; in percent) and dry biomass yield (BY; in milligrams). We selected the above-mentioned traits owing to the high extent of midparent heterosis reported in previous studies for the cross Col-0 x C24 (BARTH et al. 2003; MEYER et al. 2004).

Statistical Analyses: Means of all generations (P1, P2, F1-a, F1-b, NIL1-s, NIL2-s, NIL1-st, NIL2-st, P1 × NIL1-s, P1 × NIL2-s, P1 × NIL1-st, P1 × NIL2-st, P2 × NIL1-s, P2 × NIL2-s, P2 × NIL1-st, P2 × NIL2-st, F1-a × NIL1-s, F1-b × NIL2-s, F1-a × NIL1-st, F1-b × NIL2-st) were calculated as best linear unbiased estimates together with corresponding standard errors in a mixed model analysis of the split-plot experiment with SAS PROC MIXED (SAS INSTITUTE 2004). Error variances for the main and sub-plots were calculated as combined estimates across all types of progenies. Heritability (h²) was estimated as $h^2 = 100 \times \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2/r}$, where $\sigma_g^2$ corresponds to the genetic variance pooled across all types of progenies, $\sigma_e^2$ = variance of main-plot error + sub-plot error, and r corresponds to the number of replicates. This is an approximate estimate of heritability given that the design involved incomplete blocks (PIEPHO and MÖHRING 2007). In addition, we calculated the coefficient...
of variation (CV) as \( CV = 100 \times \frac{\sigma_e}{\overline{x}} \), where \( \overline{x} \) is the overall mean of all entries. Phenotypic \( r_p \) correlations among traits were calculated with SAS PROC CORR (SAS INSTITUTE 2004).

Means of generations were used to estimate the genetic parameters defined in Eq. 2 in a mixed model with replicates, main plots and subplots as random effects and all other effects taken as fixed. Model 1 was defined as \( G = \mu + w c + x [a] + y [d] + z [aa] + x_s a_s + y_s d_s + z_s [aa_s] + x_t a_t + y_t d_t + z_t [aa_t] \), where \( s \) and \( t \) denote the particular introgression segments as opposed to individual QTL effect in equation [1] and [2]. For Model 2, additive x additive digenic epistasis \( [aa_s t] \) between segments \( s \) and \( t \) was additionally included: \( G = \mu + w c + x [a] + y [d] + z [aa] + x_s a_s + y_s d_s + z_s [aa_s] + x_t a_t + y_t d_t + z_t [aa_t] + z_{st} aa_{st} \). The lack-of-fit of each model was tested using Wald F-tests with denominator degrees of freedom approximated by the method of KENWARD and ROGER (1997). Furthermore, models with the same fixed effects, but nested variance structure were compared by a likelihood ratio test.

The genetic parameters of Model 1 and 2 were estimated as a solution of the normal equations: \( \hat{\beta} = (X'V^{-1}X)^{-1} X'V^{-1}Y \), where \( \hat{\beta} \) is the column vector of the estimated genetic parameters; \( X \) is the design matrix with elements determined by the genetic model; \( V \) is the variance-covariance matrix of \( Y \); and \( Y \) is the vector of original phenotypic observations. The variance-covariance matrix \( V \) was replaced by an estimate determined by the restricted maximum likelihood (REML) method implemented in SAS. Standard errors of the genetic parameter estimations were calculated as the square root of the diagonal elements of matrix \( (X'V^{-1}X)^{-1} \). Significance tests of the genetic effects were performed with a Wald t-test. In addition, a sequential Bonferroni correction of P values was applied according to HOLM (1979). Significance tests for additive effects \( a_s \), dominance effects \( d_s \) and additive x additive digenic effects \( [aa_s] \) across all segments and \( aa_{st} \) between pairs of segments \( s \) and \( t \) were also performed with a Wald t-test.
RESULTS

Characterization of NIL library: We randomly sampled a subset of 15 one-segment NILs from the NIL library established by TÖRJÉK et al. (2008) (Supplementary Figure S1). The 15 one-segment NILs with the background of parent C24 or Col-0 covered a total of 31.8% of the Arabidopsis genome (Supplementary Figure S1). The length of the introgressed segments ranged between 2.5 and 22.5 cM with an average of 11.6 cM. Segments overlapped at chromosomes 1, 3, 4, and 5. These overlaps and gaps must be kept in mind when interpreting the QTL results from our study.

A sub-sample of the two-segment NILs was produced repeatedly (Supplementary Figure S1). On average, replicates derived from the same cross of one-segment NILs differed for 2.6 cM. Phenotyping revealed no significant differences among replicates of the same cross (Supplementary Table S3). Moreover, contrasts of two-segment NILs and parents were also not different for replicates of the same cross (data not shown). Therefore, replicates of the same cross were treated as the same genotype for further analyses.

Quantitative genetic variation for growth-related traits: In the analyses across all entries, estimates of genetic variances were significantly greater than zero (P < 0.05) for all traits (Table 1). To detect the fraction of variation that is genetically determined and can potentially be mapped to QTL, we estimated heritability for all seven traits as described in Material and Methods. Heritabilities ranged between 0.18 for DMC and 0.92 for RD29. Heritability was lower for DMC compared to previous results with NILs of the same cross (MELCHINGER et al. 2007a). This can be explained by a selection of NILs with relatively low genetic variation in the present survey. The coefficient of variation was highest (30.1%) for LA29 and lowest (2.7%) for DMC. Phenotypic correlations among RD22, RD29, LA22, LA29, GR, and BY were positive and high, ranging from 0.37 to 0.95 (Table 2). In contrast, moderately low
also negative correlations were observed between DMC and the other six traits, which can be explained by the low coefficient of variation for DMC.

**Net QTL effects:** Cytoplasmic effects (c) were significant (P < 0.01) for all traits except DMC and exceeded 10% of the mean μ for LA22, LA29, and BY (data not shown). The generation means analyses based on the TTC design with NILs provided an opportunity to estimate the net additive [a], dominance [d], and additive x additive [aa] effects contributing to quantitative genetic variation for growth-related traits in Arabidopsis. Net effects over the entire genome may sum up to zero, because QTL effects with positive and negative sign can cancel in the sum. Nevertheless, [a] and [d] effects were significantly (P < 0.05) greater than zero for all traits. Moreover, [aa] was significantly (P< 0.05) greater than zero for LA29 and DMC.

**Main effect QTL and additive x additive background interactions:** Our final goal was to evaluate the strategy to (1) select in a first step genomic regions with high additive x additive background interactions and (2) investigate in a second step particular additive x additive di-genic epistatic effects with two-segment NILs. Therefore, we first mapped a, d, and [aa] effects with Model 1 ignoring particular digenic epistatic aa_{st} effects. For all traits except GR, significant (P < 0.05) a, and d, effects were detected (Figure 1). Using a sequential Bonferroni correction (HOLM 1979) resulted in a decrease in the number of significant a, effects from 18 to 6 and a reduction in the number of significant d, effects from 25 to 4. Significant QTL for growth-related traits mapped to chromosomes 1, 3, 4, and 5. QTL for several traits were found at the same particular chromosomal regions.

For all traits, significant (P < 0.05) additive x additive effects of particular segments with the genetic background [aa,] were observed (Figure 1). From the 22 significant [aa,] effects, only one was negative. Means of [aa,] effects were two-times greater than those of d,
effects (Figure 2; Supplementary Figure S2). Using a sequential Bonferroni correction (HOLM 1979) resulted in a decrease in the number of significant [aaₜ] effects from 22 to 10.

Additive x additive digenic epistatic effects: Model 1 (*i.e.*, neglecting additive x additive digenic epistatic effects aaₜ) yielded a significantly (*P* < 0.01) poorer fit to the observed data than Model 2 for all traits except for GR and DMC. This clearly points to the significant role of particular additive x additive digenic epistatic effects for growth-related traits. In accordance with this expectation, 17% of the total of 98 tested aaₜ effects were significant (*P* < 0.05) (Figure 1) using Model 2. About one-third of significant aaₜ effects were negative. Using a sequential Bonferroni correction resulted in a decrease in the number of significant aaₜ effects from 17 to 9.
DISCUSSION

Epistasis is believed to be a major factor underlying quantitative traits (CAICEDO et al. 2004). The power of a particular approach to identify epistatic QTL will depend on the type of interaction and their relative magnitude (JANNINK 2007). In autogamous species such as Arabidopsis, it is expected that additive x additive epistasis is of greatest importance compared to epistasis involving dominance terms (e.g., COCKERHAM 1984). Therefore, we restricted our model to additive x additive epistasis. Presence of other types of digenic and/or higher-order epistasis, however, may lead to slightly biased estimates of genetic effects.

In terms of detectable QTL effects in NIL populations, our model is unique in that it allows the identification of (1) segments exhibiting significant additive x additive effects with the genetic background [aaₙ] and (2) particular additive x additive digenic effects aaₙst [Eq. 2]. These unique properties are required for the proposed two-step procedure to detect interacting QTL with NILs. Application of our approach is not restricted to autogamous species but is also applicable to allogamous species. Prerequisites are the availability of homozygous inbred lines and a respective library of NILs. However, for allogamous species displaying a high degree of heterosis for plant vigor such as maize, special care must be taken in the choice of the experimental design to avoid unequal competition between entries with different levels of inbreeding (e.g. lines (F=1) versus crosses (F=0) and backcrosses (F=0.5)). A solution might be a split plot design as described in MELCHINGER et al. (1986).

Two-step procedure to map epistatic QTL:

We propose to use additive x additive [aaₙ] effects with the genetic background to select parental one-segment NILs. Tests for presence of significant [aaₙ] effects can be performed efficiently by phenotyping one-segment NILs and their TTC progenies (MELCHINGER et al. 2007a). For instance, using the genome-wide Arabidopsis NIL library of TÖRJÉK et al.
results in 315 entries (78 NILs, their 234 TTC progenies, P1, P2, and F1), which have to be evaluated.

Significant $\text{aa}_s$ effects are a good indicator for the presence of digenic epistatic $\text{aa}_s$ effects, if $\text{aa}_s$ effects are unidirectional and positive and negative effects do not cancel each other in the sum [Eq. 1]. Although $\text{aa}_s$ effects detected in our study were not fully unidirectional (Figure 2; Supplementary Figure S2), for 70% of the significant $\text{aa}_s$ effects at least one significant $\text{aa}_s$ effect was detected (Figure 1). Across the seven traits, 21% of the tested segments showed a significant ($P < 0.05$) $\text{aa}_s$ effect (Figure 1) and, thus, the observed rate of 70% cannot be explained by chance alone. For about one third of the significant $\text{aa}_s$ effects, $\text{aa}_s$ effects were significant for both parental segments. For instance for BY, $\text{aa}_s$ effect of NIL1-2 and NIL1-9 were both significantly different from zero and showed agreement in sign and magnitude with the corresponding $\text{aa}_s$ effect. Thus, based on results of this study the proposed two-step procedure holds great promise for mapping particular additive x additive digenic epistatic effects but further experimental data must be gathered to corroborate its ultimate value.

**QTL analysis with NILs versus RILs:**

For Arabidopsis, the power of QTL mapping based on NILs and RILs has been compared empirically for developmental (KEURENTJES et al. 2007) and growth-related traits (MELCHINGER et al. 2007a). In both studies, QTL detection of main effects was higher for NILs than for RILs taking the population size into account. In accordance with the above results, detection power was also higher in the present study compared to the RIL experiment reported by KUSTERER et al. (2007). For overlapping chromosomal regions and traits (all except LA22 and LA29), two-times more significant ($P < 0.05$ applying sequential Bonferroni correction) main effect QTL were detected with NILs than RILs despite the smaller popula-
tion size (N=140 versus N=702). Consequently, we could confirm previous findings that NIL populations have a higher power to detect main effect QTL compared to RILs.

Three digenic aa_{st} effects were detected in the RIL study of KUSTERER et al. (2007). Corresponding two-segment NILs were not analyzed in our study. Nevertheless, two out of these three aa_{st} effects were recovered in our study by NILs with at least one QTL region displaying a significant [aa_{s}] effect. Five significant (P < 0.05 after sequential Bonferroni correction) aa_{st} effects were detected in the present study, which were not found in the RILs. These findings clearly underline the increased power to map additive x additive digenic epistatic effects with NILs compared to RILs. This increased power can be explained by (1) a reduced number of multiple tests and (2) the segregation of multiple QTL in the heterogeneous genetic background of RILs compared to the homogeneous background of NILs (KEURENTJES et al. 2007). Summarizing, the use of genome-wide NIL libraries is a promising avenue to map additive x additive digenic epistasis.

For NIL populations the resolution of QTL mapping of genetic main and digenic epistatic effects depends on the size of the introgressed segments. In our study the resolution was on average 11.6 cM. Fine mapping of QTL can be accomplished by comparison of NILs with overlapping segments (KEARSEY 2002). On chromosome 1 for instance, NIL1-4 showed a significant a_{s} and [aa_{s}] effect for DMC while this could not be confirmed for NIL1-5 (Figure 1). This can be interpreted as an indicator that the small non-overlapping segment harbors a QTL affecting DMC.

For RILs, it is possible in principle to separate also linked QTL. This was illustrated by KEURENTJES et al. (2007) by the detection of two major QTL for flowering time detected in the RIL population on the top of chromosome 5. These QTL were linked and showed strong epistatic interactions. Nevertheless, separate detection of linked QTL is very unlikely if their effect sizes are small, because confidence intervals of point estimates of QTL positions are commonly rather wide (UTZ et al. 2000).
In QTL mapping studies based on RIL populations, model selection is crucial especially when testing for presence of epistasis (for a review see SILLANPÄÄ and CORANDER 2002). Even with a moderate number of putative QTL, including all possible epistatic effects must accommodate a very large number of potential parameters, which causes problems in determination of the genetic model (YI et al. 2003). In contrast, QTL mapping based on NILs is much less afflicted with the problem of model selection, and genetic effects are estimated for each introgressed segment. This is an additional advantage of QTL mapping based on NILs compared to RILs.

Repeatability of QTL mapping studies with NILs: It is appealing to compare our results with those of a previous study with one-segment NILs of the same cross, where $a_s$, $d_s$, and $[a_s]$ effects were investigated for the same traits with an overlap of 14 one-segment NILs (MELCHINGER et al. 2007a). Considering the overlapping chromosomal regions, 39% of $a_s$, 44% of $d_s$, and 47% of $[a_s]$ effects that were significant ($P < 0.05$) in our study were also reported by MELCHINGER et al. (2007a). One explanation of the observed discrepancy between findings of our study and those of MELCHINGER et al. (2007a) are genotype × environment interactions. This hypothesis is supported by significant genotype × environment interactions ($P < 0.01$) in the combined analyses of parents and $F_1$ hybrids across both studies (data not shown). Even though utmost care was exercised to warrant uniform temperature, light, and moisture conditions, a perfect control of the environmental conditions across both studies was not possible. Consequently, overlapping QTL will mostly include those that displayed no or little QTL × environment interactions.

QTL with strong environment dependencies may limit the usefulness of the suggested two-step approach to map digenic epistatic effects. Consequently, phenotyping should be performed in a wide range of target environments in the first phase facilitating a precise and robust estimation of the genuine genotypic effect. Furthermore, the developed models can easily
be extended for interaction terms between environments and genetic effects. This opens the possibility to test whether $a_s$, $d_s$, $[aa_s]$ and $aa_{sd}$ effects are to the same degree influenced by the target environment.

**Genetic architecture of growth-related traits in Arabidopsis:** We used TTC progenies of NILs to map $a_s$, $d_s$, $[aa_s]$, and $aa_{sd}$ effects. Separate estimation of these effects is not feasible by analyzing only NILs not including their TTC progenies. Furthermore, mapping approaches using TTC progenies yield unbiased estimates of genetic effects (MELCHINGER et al. 2008). Consequently, the applied experimental approach represents a promising strategy towards elucidating the genetic architecture of quantitative traits. Furthermore, we randomly selected the parents of the two-segment NILs analyzed in our study, which is a prerequisite to obtain unbiased estimates for the distribution of the $aa_{sd}$ effects. This is of particular interest because this sampling strategy yields an unbiased picture of predominant genetic effects. Nevertheless, a bias in estimation of genetic effects cannot fully be ruled out due to replicated sampling of overlapping one-segment NILs.

Significant effects of chromosomal segments on trait performance can be due to (1) one QTL or (2) several QTL linked in coupling phase (KEURENTJES et al. 2007). The probability to detect single QTL increases when investigating smaller introgression segments or by comparing overlapping segments (ESHED and ZAMIR 1995). Nevertheless, experimental data for growth rate in Arabidopsis showed that even for small chromosome intervals of 1cM more than one QTL is possible (KROYMANN and MITCHELL-OLDS 2005). Therefore, we cannot determine whether single QTL or several QTL linked in coupling phase underlie the genetic effects detected in our study (Figure 1). Consequently, identified QTL effects have to be interpreted as compound effects of particular chromosomal segments.

The observed clustering of QTL for different traits (Figure 1) was not unexpected given the strong correlations observed among growth-related traits except for DMC (Table 2). The
clustering of effects can be explained by (1) different QTL linked on one introgression segment or (2) pleiotropic effects of single QTL. High resolution mapping is required to determine whether pleiotropic effects are present. Interestingly, for the introgressed segments on chromosome 3 we observed only QTL for LA and RD determined 22 days after sowing (DAS) but not at 29 DAS. This is a strong indicator for the dynamic nature of the traits under consideration with genes and gene networks active during different phases of plant development.

For all traits absolute values of $a_s$ effects were larger than those of $d_s$ effects (Figure 2; Supplementary Figure S2). Consequently, additive effects are a major component for complex trait formation. In addition, sizes of $[aa_s]$ effects were comparable to the magnitude of $a_s$ effects (Figure 2; Supplementary Figure S2). This is in accordance with previous results in Arabidopsis (KUSTERER et al. 2007) and clearly underlines the importance of epistasis for complex trait formation. If we consider Arabidopsis as a model species for autogamous crops, then the relevance of $[aa_s]$ effects suggests difficulties in marker assisted selection, because genetic values of introgressed segments are strongly influenced by the genetic background of the recipient. Nevertheless, for each trait the maximum $aa_{st}$ effect was larger than the 75% quartile of genetic main effects except for DMC. This suggests that particular additive x additive digenic interactions are relevant and can be exploited in marker assisted selection programs only by introgression of complementary segments exhibiting positive digenic epistasis.
We thank O. Törjék for the SNP marker assays. We gratefully acknowledge the expert technical assistance of B. Devezi-Savula, N. Friedl, C. Marona, M. Teltow, M. Zeh, and M. Zehnsdorf. This project was supported by Deutsche Forschungsgemeinschaft (German Research Foundation) under priority research program “Heterosis in Plants” (research grants AL387/6-1, AL387/6-2, ME931/4-1, ME931/4-2, PI377/7-1, PI377/7-2, RE2254/1-1, and RE2254/1-2).
LITERATURE CITED


ESHED, Y., and D. ZAMIR, 1995 An Introgression line population of Lycopersicon pennellii in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141: 1147-1162.


JANSEN, R. C., 1993 Interval mapping of multiple quantitative trait loci. Genetics 135: 205-211.


KUSTERER, B., H. P. PIEPHO, H. F. UTZ, C. C. SCHÖN, J. MUMINOVIC et al., 2007 Heterosis for biomass-related traits in Arabidopsis investigated by QTL analysis of the triple test cross design with recombinant inbred lines. Genetics 177: 1839-1850.


LYNCH, M., and B. WALSH, 1998 Genetics and analysis of quantitative traits. Sinauer, Sunderland, Massachusetts, USA


MELCHINGER, A. E., H. F. UTZ and C. C. SCHÖN, 2008 Genetic expectations of quantitative trait loci main and interaction effects obtained with the triple testcross design and their relevance for the analysis of heterosis. Genetics 178: 2265-2274.


TABLE 1

Means (\(\bar{X}\)), genotypic variance component (\(\sigma^2_g\)), sum of main- and sub-plot error variance (\(\sigma^2_e\)), broad sense heritability (\(h^2\)), and coefficient of variation (CV) for seven growth-related traits for the 140 genotypes used in the study

<table>
<thead>
<tr>
<th>Trait</th>
<th>(\bar{X}) ± (SE)</th>
<th>(\sigma^2_g)</th>
<th>(\sigma^2_e)</th>
<th>(h^2)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD22 (mm)</td>
<td>39.5 (0.7)</td>
<td>36.8 **</td>
<td>21.9 **</td>
<td>0.83</td>
<td>15.37</td>
</tr>
<tr>
<td>RD29 (mm)</td>
<td>92.1 (1.4)</td>
<td>324.9 **</td>
<td>79.0 **</td>
<td>0.92</td>
<td>19.57</td>
</tr>
<tr>
<td>GR (mm/d)</td>
<td>7.5 (0.2)</td>
<td>2.1 **</td>
<td>0.9 **</td>
<td>0.87</td>
<td>19.15</td>
</tr>
<tr>
<td>LA22 (mm²)</td>
<td>624.9 (24.4)</td>
<td>33564.0 **</td>
<td>21136.0 **</td>
<td>0.83</td>
<td>29.32</td>
</tr>
<tr>
<td>LA29 (mm²)</td>
<td>3417.2 (85.0)</td>
<td>1060407.0 **</td>
<td>404560.0 **</td>
<td>0.89</td>
<td>30.13</td>
</tr>
<tr>
<td>DMC (%)</td>
<td>8.5 (0.6)</td>
<td>0.1 *</td>
<td>0.7 **</td>
<td>0.18</td>
<td>2.65</td>
</tr>
<tr>
<td>BY (mg)</td>
<td>106.0 (9.3)</td>
<td>581.5 *</td>
<td>649.3 **</td>
<td>0.73</td>
<td>22.75</td>
</tr>
<tr>
<td></td>
<td>RD22</td>
<td>RD29</td>
<td>GR</td>
<td>LA22</td>
<td>LA29</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>RD22</td>
<td>0.77*</td>
<td>0.42*</td>
<td>0.95*</td>
<td>0.79*</td>
<td>0.06*</td>
</tr>
<tr>
<td>RD29</td>
<td>0.89*</td>
<td>0.71*</td>
<td>0.94*</td>
<td>-0.11*</td>
<td>0.72*</td>
</tr>
<tr>
<td>GR</td>
<td>0.37*</td>
<td>0.78*</td>
<td>-0.22*</td>
<td>0.48*</td>
<td></td>
</tr>
<tr>
<td>LA22</td>
<td></td>
<td>0.76*</td>
<td>0.04</td>
<td>0.77*</td>
<td></td>
</tr>
<tr>
<td>LA29</td>
<td></td>
<td></td>
<td>0.06</td>
<td>0.83*</td>
<td></td>
</tr>
<tr>
<td>DMC</td>
<td></td>
<td></td>
<td></td>
<td>0.38*</td>
<td></td>
</tr>
</tbody>
</table>

*, ** significantly different from zero at P < 0.05 and P < 0.01, respectively.
FIGURE 1. Additive ($a_i$), dominance ($d_i$), and additive × additive effects of a single segment with the genetic background ($[a_{aa}]$), as well as specific digenic additive × additive effects ($aa_{aa}$) for seven growth-related traits in Arabidopsis thaliana. Values followed by * or ** are significant at the probability level of $P < 0.05$ and $P < 0.01$, respectively.
Rosette diameter at 22 days after sowing

Chromosome 1  Chromosome 2  Chromosome 3  Chromosome 4  Chromosome 5

Effect NIL1-1 NIL2-1 NIL1-2
\[ a_s = 6.1^* \]  \[ a_s = -5.8 \]  \[ a_s = 7.9^{**} \]  aa_{st} = -0.4
\[ d_s = 2.6^* \]  \[ d_s = 4.1^* \]  \[ d_s = 3.9^* \]  aa_{st} = -1.0
\[ [a_{st}] = 4.5^* \]  \[ [a_{st}] = 6.4^* \]  \[ [a_{st}] = 8.2^{**} \]  aa_{st} = 4.3^{**}

Effect NIL2-2
\[ a_s = -1.9 \]  \[ d_s = 0.5 \]  \[ [a_{st}] = 2.9 \]
\[ a_{st} = 3.6^* \]  \[ a_{st} = 0.4 \]  aa_{st} = -0.7

Effect NIL1-3
\[ a_s = -0.4 \]  \[ d_s = -0.4 \]  \[ [a_{st}] = -1.7 \]
\[ a_{st} = -1.4 \]  aa_{st} = -1.3

Effect NIL1-4 NIL1-5
\[ a_s = -2.2 \]  \[ d_s = -0.6 \]  \[ [a_{st}] = -2.9 \]
\[ a_{st} = -1.9 \]  aa_{st} = -1.1

Effect NIL2-3 NIL1-6
\[ a_s = 0.5 \]  \[ d_s = 0.5 \]  \[ [a_{st}] = 0.5 \]
\[ a_{st} = -1.6 \]  aa_{st} = -2.9

Effect NIL1-7 NIL1-8
\[ a_s = 5.3 \]  \[ d_s = -0.9 \]  \[ [a_{st}] = 4.8 \]
\[ a_{st} = -1.9 \]  aa_{st} = -2.8

Effect NIL2-9
\[ a_s = -3.2 \]  \[ d_s = 3.8^{**} \]  \[ [a_{st}] = 3.7^{**} \]
\[ a_{st} = 4.0^{**} \]  \[ a_{st} = -3.9^{*} \]  aa_{st} = 2.0

Effect NIL1-9
\[ a_s = -2.5 \]  \[ d_s = -0.9 \]  \[ [a_{st}] = 4.3^{*} \]
\[ a_{st} = -1.9 \]  aa_{st} = -2.3^{*}

Effect NIL1-10 NIL2-4 NIL2-5
\[ a_s = 6.6^* \]  \[ d_s = -11.0^{**} \]  \[ d_s = -5.1 \]  \[ aa_{st} = 4.0 \]
\[ d_s = 6.8^{*} \]  \[ aa_{st} = -2.9^{*} \]  aa_{st} = 2.2

Rosette diameter at 29 days after sowing
Leaf area at 22 days after sowing

Chromosome 1  Chromosome 2  Chromosome 3  Chromosome 4  Chromosome 5

Effect NIL1-1 NIL2-1 NIL1-2
\( a_s \)  180+  -74  219+  \\
\( d_s \)  79  99  112+  \\
\([a d_a]\)  120  113  231**

Effect NIL1-6
\( a_s \)  -10  \\
\( d_s \)  9  \\
\([a d_a]\)  -6

Effect NIL1-3
\( a_s \)  -28  \\
\( d_s \)  -10  \\
\([a d_a]\)  -57

Effect NIL2-2
\( a_s \)  -40  \\
\( d_s \)  -2  \\
\([a d_a]\)  70

Effect NIL2-3 NIL1-9
\( a_s \)  96*  95  \\
\( d_s \)  -10  \\
\([a d_a]\)  43  133*

Effect NIL1-7 NIL1-8
\( a_s \)  96*  10  \\
\( d_s \)  99*  -65*  \\
\([a d_a]\)  98  -36

Effect NIL2-3 NIL1-9
\( a_s \)  237*  -335** -130  \\
\( d_s \)  36  89*  34  \\
\([a d_a]\)  157  230**  63

aa_{st} = -11

aa_{st} = -11

aa_{st} = 141**

aa_{st} = -41

aa_{st} = -26

aa_{st} = -48

aa_{st} = -67

aa_{st} = -66*
Leaf area at 29 days after sowing
Dry matter content

Chromosome 1  Chromosome 2  Chromosome 3  Chromosome 4  Chromosome 5

Effect NIL1-1 NIL2-1 NIL1-2
\( a_s \) 1.2** -0.7 0.0
\( d_s \) 0.4** -0.1 -0.1
[\( a_d \)] 0.9** 0.6 0.1

Effect NIL2-2
\( a_s \) -0.1
\( d_s \) 0.3
[\( a_d \)] 0.3

Effect NIL1-3
\( a_s \) 0.1
\( d_s \) 0.1
[\( a_d \)] 0.1

Effect NIL1-6
\( a_s \) 0.4
\( d_s \) 0.1
[\( a_d \)] 0.3

Effect NIL1-10 NIL2-4 NIL2-5
\( a_s \) 1.1** -0.8** -0.7
\( d_s \) 0.3 0.2 0.2
[\( a_d \)] 0.7** 0.7** 0.5

Effect NIL1-4 NIL1-5
\( a_s \) -1.4** -0.8
\( d_s \) -0.4 -0.0
[\( a_d \)] -0.9** -0.0
FIGURE 2. Boxplots of additive ($a_s$), dominance ($d_s$), additive $\times$ additive effects of a single segment with the genetic background ([aa,]), as well as specific digenic additive $\times$ additive effects (aa_a) for dry biomass yield. The closed boxes comprise values between the 25% and 75% quantiles.