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ORIGINAL PAPER

Multi-trait association mapping in sugar beet (*Beta vulgaris* L.)

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Abstract Association mapping promises to overcome the limitations of linkage mapping methods. The main objective of this study was to examine the applicability of multivariate association mapping with an empirical data set of sugar beet. A total of 111 diploid sugar beet inbreds was selected from the seed parent heterotic pool to represent a broad diversity with respect to sugar content (SC). The inbreds were genotyped with 26 simple sequence repeat markers chosen according to their map positions in proximity to previously identified quantitative trait loci for SC. For SC and beet yield (BY), the genotypic variances were highly significant ($P < 0.01$). Based on the global test of the bivariate mixed-model approach, four markers were significantly associated with SC, BY, or both at a false discovery rate of 0.025. All four markers were significantly ($P < 0.05$) associated with BY but only two with SC. The identification of markers associated with SC, BY, or both indicated that association mapping can be successfully applied in a sugar beet breeding context for detection of marker-phenotype associations. Furthermore, based on our results multivariate association mapping can be recommended as a promising tool to discriminate with a high mapping resolution between pleiotropy and linkage as reasons for co-localization of marker-phenotype associations for different traits.

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Introduction

Sugar beet (*Beta vulgaris* L.) accounts for about 25% of the worldwide sugar production (Draycott 2006). Sugar content (SC) and beet yield (BY) determine sugar yield (SY) and, thus, are of major economic importance and under high selection pressure in breeding programs (Schneider et al. 2002). Knowledge of the genetic architecture of such traits would facilitate sugar beet breeding (Holland 2007).

In sugar beet, linkage mapping was employed to dissect quantitative traits in the underlying quantitative trait loci (QTL). Weber et al. (1999, 2000) analyzed QTL for SY and its components in two segregating populations grown in different locations. Schneider et al. (2002) identified QTL for SY and quality parameters in a segregating population using expressed sequence tag related markers.

Major limitations of linkage mapping approaches are (1) poor resolution in detecting QTL and (2) sampling of only two alleles at any given locus in biparental crosses of inbred lines (Flint-Garcia et al. 2003). Association mapping methods, which were successfully applied in human genetics to detect genes coding for human diseases (cf., Lowe et al. 2007), promise to overcome these limitations (Kraakman et al. 2004). Therefore, in plant genetics several attempts have been made for detecting QTL by such methods (e.g.,

Breseghello and Sorrells 2006; Wilson et al. 2004). Furthermore, it is rather appealing to plant breeders to exploit for association mapping those genotypes that were evaluated in the framework of routine plant breeding trials. To our knowledge, however, no study investigated the applicability of this mapping strategy in a sugar beet breeding context.

In most association mapping studies, data were collected for several traits, but analysed separately for each trait (e.g., Breseghello and Sorrells 2006; Wilson et al. 2004). Thus, it is not possible to discriminate between pleiotropy and linkage of genes as underlying causes of genetic correlation between traits. Consequently, only partial information about the genetic architecture of the traits under consideration is revealed (Rencher 1998). This limitation can be overcome by applying multi-trait statistical methods as suggested for linkage mapping of QTL by various authors (e.g., Jiang and Zeng 1995; Korol et al. 1995). Nevertheless, to our knowledge no study investigated such approaches in an association mapping context.

The objective of our research was to examine the applicability of multivariate association mapping to discriminate between pleiotropy and linkage as reasons for co-localization of marker-phenotype associations for different traits with an empirical data set of sugar beet.

Materials and methods

Plant materials, field experiments, and molecular markers

Our study was based on 111 diploid sugar beet inbreds which were selected from the seed parent heterotic pool to represent a broad diversity with respect to SC. For these inbreds which did not carry a monogenic resistance against rhizomania, pedigree information was available up to three generations back (Electronic supplementary material S1). Testcross progenies were produced by crossing the 111 inbreds to one tester of the pollen parent heterotic pool. All plant materials used in this study are proprietary to KWS SAAT AG (Einbeck, Germany).

In 2003, the 111 testcross progenies were evaluated in routine plant breeding trials (α-lattice designs) with three replications at six locations in Germany. The soil of location 1–3 was infested with beet necrotic yellow vein virus, the causal agent of rhizomania (Tamada and Baba 1973), whereas the soil of location 4–6 was virus free. Data were recorded for SC and BY in % of the mean performance of four checks based on lattice-adjusted entry means.

All 111 sugar beet inbreds were fingerprinted by KWS SAAT AG according to standard protocols with 26 simple sequence repeat (SSR) markers. The markers were selected according to their map positions in proximity to previously identified QTL for SC (KWS SAAT AG, unpublished data) and cover about 20% of the sugar beet genome (cf., Schumacher et al. 1997). Map positions of all markers were based on the linkage map of KWS SAAT AG (Electronic supplementary material S2).

Statistical analyses

The combined analysis of adjusted entry means across locations does not allow to make inferences about entry × location interactions (cf., Piepho 2000). Nevertheless, the results of Stich et al. (2008a) indicated that two-step association approaches based on adjusted entry means for each location posses only a slightly reduced power for detection of marker-phenotype associations than one-step approaches. Therefore, our analyses were based on adjusted entry means calculated for each location. A hierarchical cluster analysis was performed on the correlation coefficient of adjusted entry means among all pairs of locations to examine the presence of distinct subgroups of locations.

Bivariate phenotypic data analysis

The combined analysis of adjusted entry means across locations was performed based on the bivariate statistical model:

$$y_{ijt} = \mu_t + g_{it} + l_{jt} + e_{ijt}, \quad (1)$$

where y_{ijt} is the adjusted entry mean of the ith sugar beet inbred at the jth location for the tth trait, μ_t the intercept term for the tth trait, g_{it} the genetic effect of the ith sugar beet inbred for the tth trait, l_{jt} the effect of the jth location for the tth trait, and e_{ijt} the residual. Because locations were purposefully selected, l_{jt} was regarded as fixed, whereas g_{it} was regarded as random.

The random variables g and e were assumed to be bivariate normally distributed (BVN) and mutually uncorrelated. Specifically, g was BVN ($\mathbf{0}$, $\mathbf{G} \otimes \mathbf{A}$) and e was BVN ($\mathbf{0}$, $\mathbf{E} \otimes \mathbf{R}$). Matrices $\mathbf{G}$ and $\mathbf{E}$ included variances and covariances among the traits due to genetic and residual effects, respectively. The symbol $\otimes$ represents the Kronecker product. $\mathbf{A}$ was a 111 × 111 matrix of coancestry coefficients between all pairs of sugar beet inbreds. Coancestry coefficients (f) were calculated based on the available pedigree records, according to the rules described by Falconer and Mackay (1996) and using PROC INBREED in SAS (SAS Institute 2004). $\mathbf{R}$ was a 666 × 666 identity matrix.

Bivariate association analysis

In studies based on testcross progenies with a common tester, no dominance effects can be estimated, because the

allele effects a comprise also the dominance effects between parental alleles and those of the tester (Melchinger 1988).

Therefore, we used the following statistical model for bivariate association analysis:

$$y_{ijpt} = \mu_t + a_{pt} + g^*_{it} + l_{jt} + (al)_{jpt} + e_{ijpt}, \quad (2)$$

where y_{ijpt} is the adjusted entry mean of the ith sugar beet inbred at the jth location carrying allele p for the tth trait, a_{pt} the effect of allele p for the tth trait, g^*_{it} the genetic effect of the ith inbred except for a_{pt}, $(al)_{jpt}$ the interaction effect of the pth allele with the jth location for the tth trait, e_{ijpt} the residual. In addition to l_{jt}, we regarded a_{pt} and $(al)_{jpt}$ as fixed, whereas g^*_{it} was regarded as random. The assumptions made in the bivariate phenotypic data analysis concerning the random variables g and e were also made in this analysis for g^* and e.

Based on the Wald F statistic, we performed in a first step a global test for the presence of a marker-phenotype association with an effect on SC, BY, or both. Different procedures can be applied to correct for multiple testing. The results of Chen and Storey (2006) suggested that the false discovery rate (FDR) procedure proposed by Storey and Tibshirani (2003) was inappropriate to correct for multiple testing in a linkage mapping context. In our study, however, the genetic map distance in which linkage disequilibrium decays is expected to be considerably lower than the average marker distance and, thus, the use of the abovementioned FDR procedure is valid. Therefore, marker-phenotype associations for which the FDR q value was <0.025 were considered significant. The same procedure was used for detecting significant marker × location interactions, which correspond to QTL × location interactions in a linkage mapping context (e.g., Piepho 2000). At positions, where the global test indicated a marker-phenotype association, we tested the specific effects on SC and BY by a Wald F test. Following Malosetti et al. (2007), we regarded tests with $P < 0.05$ as significant. The total proportion of the genotypic variance explained by all markers with significant main effect was obtained by fitting a model including all these markers simultaneously.

All mixed-model calculations were performed with ASReml release 2.0 (Gilmour et al. 2006).

Results

Significant ($P < 0.01$) genotypic variance was observed for SC as well as BY (Table 1). Residuals showed no significant ($P < 0.05$) deviation from a normal distribution. Heritability on an adjusted entry mean basis was high for SC (0.97) and BY (0.92). The genetic correlation between SC and BY was −0.72, whereas the residual correlation was −0.11. The cluster analysis based on the correlation coefficient of adjusted entry means among all pairs of locations revealed no consistent patterns of subgroups of locations for SC and BY (Fig. 1).

Table 1 Second degree statistics for sugar content (SC) and beet yield (BY) of sugar beet testcross progenies in percentage of the mean performance of four checks

Parameter	SC (%)		BY (%)
σ^2_g	8.44**		30.67**
ρ_g		−0.72	
σ^2_e	1.77		15.62
ρ_e		−0.11	
h^2	0.97		0.92

σ^2_g and σ^2_e are the genotypic and error variances, respectively. ρ_g and ρ_e are the genotypic and residual correlations, respectively. h^2 is the heritability on an adjusted entry mean basis

** Significant at $P < 0.01$

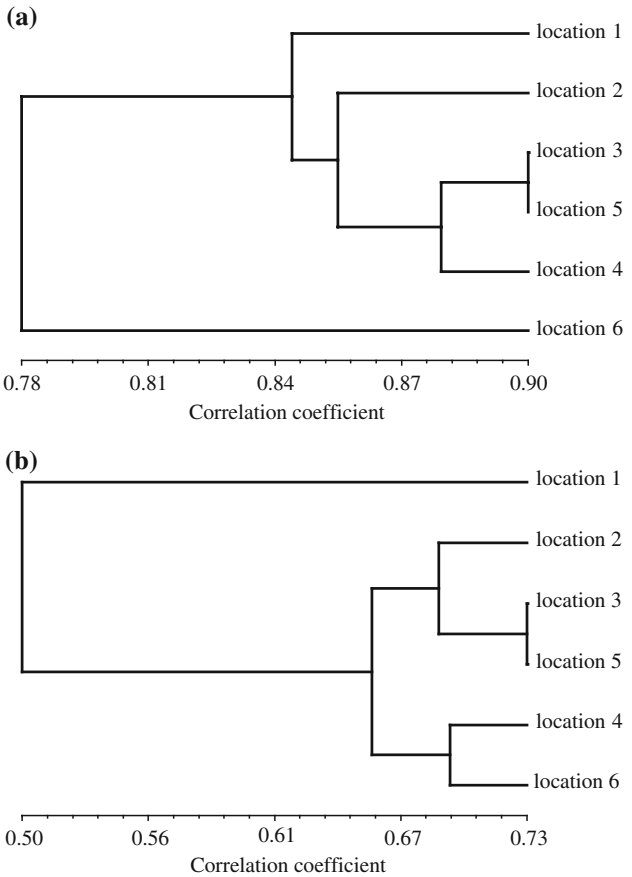

Fig. 1 Dendrogram of the correlation coefficient of adjusted entry means among all pairs of locations calculated for **a** sugar content and **b** beet yield. The soil of location 1–3 was infested with beet necrotic yellow vein virus, the causal agent of rhizomania, whereas the soil of location 4 to 6 was virus free

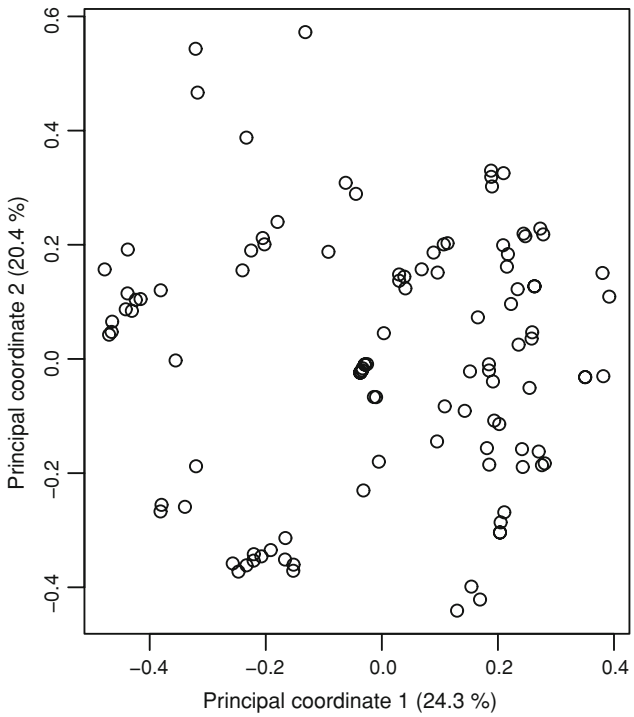

Fig. 2 Principal coordinate analysis of 111 sugar beet inbreds of the seed parent germplasm group based on $1-f$. Number in parentheses refer to the proportion of variance explained by the principal coordinate

The total number of alleles detected for the 26 SSR markers was 85, with the number of alleles per locus ranging from two to five. The allele frequency varied between 0.01 and 0.94 (Electronic supplementary material S2). Estimates of f between all pairs of sugar beet inbreds ranged from 0.00 to 0.62 with an average of 0.07. In principal coordinate analysis based on $1-f$ between all pairs of sugar beet inbreds, the first two principal coordinates explained 24.3 and 20.4% of the molecular variance (Fig. 2). With respect to these two principal coordinates, no distinct sub-populations were detected.

Based on the global test of the bivariate mixed-model approach, four markers were significantly ($q < 0.025$) associated with SC, BY, or both (Table 2). All four markers were significantly ($P < 0.05$) associated with BY but only two with SC. The proportion of the genotypic variance explained by the markers for SC ranged from 0.23 to 1.63%, whereas for BY the proportion ranged from 1.94 to 7.09%. The proportion of the genotypic variance explained simultaneously by all markers with significant main effect was 1.74% for SC and 20.29% for BY.

The two markers associated with SC as well as BY explained 4.09 and 5.41% of the genetic covariance between these traits. None of the 26 markers displayed significant ($q < 0.025$) marker × location interactions.

Discussion

Statistical approach for association mapping

The presence of population structure (Flint-Garcia et al. 2003) as well as familial relatedness (Stich et al. 2005) in a germplasm set can result in a type I error rate for association mapping which is considerably higher than the nominal α level. The current study, however, was based on inbreds from one heterotic group. This fact is expected to largely eliminate population structure as linkage disequilibrium generating force. In our study, the complex familial relatedness of the examined sugar beet inbreds (Electronic supplementary material S1) was considered by applying a mixed-model association mapping method in which the degree of genetic covariance between all pairs of sugar beet inbreds was accounted for by the matrix of coancestry coefficients calculated from pedigree records. Therefore, no inflated rate of false positive marker-phenotype associations is expected.

Multi-trait association mapping

In most linkage mapping studies, data from several traits were collected and series of single-trait analyses were performed for QTL detection (e.g., Schön et al. 2004). Such trait-by-trait analyses, however, may overlook important information for plant breeders (Hackett et al. 2001). Because the same argument applies to association mapping, we employed a multi-trait association mapping approach.

In a linkage mapping context, Weller et al. (1996) and Mangin et al. (1998) proposed QTL detection for a set of correlated traits by mapping their uncorrelated principal components. Such an analysis, however, might detect spurious QTL, when applied to traits with a mixture of genetic and environmental correlation (Szyda et al. 2003). Therefore, the analysis of principal components was discarded for the traits examined in our study (Table 1). In contrast, multivariate statistical analyses should be appropriate for any combination of environmental and genetic correlation (Hackett et al. 2001).

The results of Jiang and Zeng (1995) suggested that in a linkage mapping context, the power for QTL detection of bivariate analyses can increase significantly in comparison with separate analyses of each trait if the relevant QTL has pleiotropic effects on both traits with the product of the effects differing in sign from the residual correlation. If the product of the pleiotropic effects and the residual correlation have the same sign, however, the test statistic of the bivariate analysis will be smaller than the sum of the test statistics under the separate tests. In this case, the power for QTL detection of the bivariate analysis may be lower than

Table 2 Marker loci significantly ($q < 0.025$) associated with sugar content (SC), beet yield (BY), or both. Data for SC and BY were recorded in % of the mean performance of four checks based on lattice-adjusted entry means. $\widehat{p_g}$ is the explained proportion of the genotypic variance and $\widehat{p_{g_{Cov}}}$ the explained proportion of the genotypic covariance between SC and BY

Marker locus	Linkage group	Position (cM)	Trait	Effect			$\widehat{p_g}$ (%)	$\widehat{p_{g_{Cov}}}$ (%)	Marker × location
				Allele 1	Allele 2	Allele 3			
M4	C	0	SC*	0	−1.15	−1.53	1.63	5.41	NS
			BY*	0	5.26	7.85	5.98		
M13	E	14	SC^{NS}						NS
			BY*	0	1.42	2.04	1.94		
M18	E	42	SC*	0	−1.05		0.23	4.09	NS
			BY*	0	2.83		7.09		
M22	G	9	SC^{NS}						NS
			BY*	0	4.00		2.84		
Total			SC				1.74	6.97	
			BY				20.29		

NS Nonsignificant

* Significant at $P < 0.05$

that of the separate analysis of each trait. Nevertheless, Jiang and Zeng (1995) found that joint analyses were generally more informative than separate analyses for moderately correlated traits. This finding might explain the fact that in our study no significant ($q < 0.025$) marker-phenotype associations were detected for SY calculated as SC × BY based on an univariate mixed-model approach (data not shown), whereas four markers were significantly ($q < 0.025$) associated with SC, BY, or both based on a bivariate approach.

Detected marker-phenotype associations

In the current study, the proportions of the genotypic variance explained by the detected markers individually as well as their simultaneous fit (Table 2) were considerably lower than the values reported by Schneider et al. (2002) for the same traits in a linkage mapping experiment of similar size in sugar beet. One explanation could be the difference in allele frequencies expected for the germplasm in both studies because the proportion of the genotypic variance explained by a marker is a function of (1) allele frequency, (2) allele effect, and (3) linkage disequilibrium between marker and QTL. Under the assumption of fixed allele effects and additive gene action, as applies to test-cross performance, the maximum of the proportion of genotypic variance explained by a marker is observed for an allele frequency of 0.5, as expected for the entries derived from a biparental cross. In contrast, for a germplasm set as examined in our study, the allele frequencies are expected to be considerably different from 0.5 especially if multi-allelic markers are examined (Electronic supplementary material S2). Thus, the proportion of the genotypic variance explained by a marker is notably lower despite the same underlying allele effect. The allele frequencies of plant breeding populations are expected to be more similar to those observed in our association mapping population than to those of a population derived from a biparental cross (Crepieux et al. 2004). Therefore, we conclude that the former approach has only a low statistical power to detect QTL which allele frequencies strongly deviating from 0.5 but this leads to a more representative estimate of the variance accounted for by a marker in the breeding population.

Another reason for the considerable discrepancy in the proportion of the explained genotypic variance observed in our study and the biparental cross of Schneider et al. (2002) ist most likely attributable to the different concepts in choosing the germplasm underlying these studies. In the present study, current elite genotypes of sugar beet were used while in the study of Schneider et al. (2002) the parents of the mapping population were chosen in such a way that they maximally differed for the examined traits. The latter approach increases the probability of detecting QTL explaining a large proportion of the genotypic variance (Lander and Botstein 1989). However, in contrast to the present study, this procedure leads to QTL information which might be of little value for marker-assisted selection in elite plant breeding programs because the favorable QTL allele might already be fixed in the elite germplasm pool.

A further explanation for the considerable discrepancy in the proportion of the genotypic variance explained by the detected markers simultaneously could be the fact that the markers of our study covered 20% of the sugar beet genome whereas the entire genome was sampled in the study of Schneider et al. (2002).

Discerning genetic linkage and pleiotropy with association mapping

A QTL with significant effects on both traits can be due to a single pleiotropic QTL or two closely linked QTL each with an effect on only one trait. In a bivariate linkage mapping context, the discrimination between both situations can be

made based on a two-dimensional scan around the QTL position (e.g., Szyda et al. 2003). The size of the genome region examined in this scan is typically between 20 and 40 cM (cf., Jiang and Zeng 1995; Malosetti et al. 2007). In this region, flanking marker genotypes are used to calculate the test statistic based on the conditional probabilities of marker genotypes (Lander and Botstein 1989).

Although the principle for discerning genetic linkage and pleiotropy with association mapping is similar to that of linkage mapping, several important differences do exist. In an association mapping context, the parental genotypes as well as the recombination history of the germplasm set under consideration is unknown, in contrast to linkage mapping studies. Hence, no conditional probabilities of marker genotypes can be estimated from flanking marker genotypes. Consequently, in association mapping studies, the two-dimensional scan is restricted to the marker loci genotyped.

The advantage of association mapping approaches is that their mapping resolution is considerably higher than that of linkage mapping approaches (Flint-Garcia et al. 2003). Therefore, the discrimination between pleiotropy and close linkage of QTL, which is hardly possible in linkage mapping approaches (Malosetti et al. 2007), is more likely feasible in association mapping approaches. This was supported by our observation that for the cluster of five markers mapping to the same genome region on linkage group E with less than 1 cM (corresponding to 1.22 $\times$ 10^6 base pairs; Arumuganathan and Earle 1991; Halldén et al. 1996), only one marker (M18) showed a significant association based on the global test (Table 2), whereas in linkage mapping studies confidence intervals for QTL positions are typically in the range of 10–20 cM.

Because association mapping approaches are restricted to the marker loci genotyped, the two dimensional scan to discern close linkage and pleiotropy is only required for genome regions in which more than one significant association was detected based on the global test. In contrast, for genome regions in which only one significant association was detected, like observed in our study for M4 and M18, no two-dimensional scan is required. For such regions, the hypothesis of pleiotropy can not be rejected.

Dissection of the genetic correlation between SC and BY

The allele effects of the markers M4 and M18, identified to be associated with both SC as well as BY, induce a negative genetic correlation between these traits. This correlation was in the same direction as expected from our phenotypic data analysis (Table 1) as well as from physiological coherences between SY and BY (Milford 1976). The two markers explained about 7% of the observed genetic covariance between SC and BY. Our findings suggested that SC and BY could not be improved concurrently by using markers M4 and M18 in a marker-assisted selection program.

The major factors influencing the power for detecting marker-phenotype associations are the (1) heritability of the phenotypic traits under consideration, (2) allele frequency of marker alleles, (3) the extent of linkage disequilibrium between marker and QTL, as well as (4) population size of the germplasm set used for association mapping. The heritability on an entry mean basis observed in our study for SC and BY was high. In addition, in an association mapping context, the allele frequency of the marker alleles can hardly be influenced. The selection of markers in proximity to previously identified QTL increases the probability of significant LD between markers and QTL. Nevertheless, the markers of our study covered only 20% of the sugar beet genome. Furthermore, the population size of our germplasm set was certainly at the lower end (cf., Stich et al. 2008b). In addition to the negative correlation expected from physiological coherences between SY and BY, these last two facts might explain the failure to detect markers which are significantly associated with SC as well as BY but induce no or a positive correlation between both traits. Because such markers are of high interest in marker-assisted selection programs, we propose to examine SC and BY based on a larger germplasm set with a higher number of markers than that used in our study to achieve a higher statistical power for detection of marker-phenotype associations.

Marker $\times$ location interactions

For the 26 markers in our study, no significant marker $\times$ location interactions were observed (Table 2). This finding is in accordance with results from most linkage mapping studies (e.g., Cockerham and Zeng 1996; Melchinger et al. 1998), which rarely found significant marker $\times$ location interactions despite the presence of significant genotype $\times$ location interactions. This result might be explained by the fact that entries were grown in a relatively small number of locations which allows no reliable estimation of marker $\times$ location interactions (Melchinger et al. 1998).

In our study, the soil of three of the examined six locations was infested with beet necrotic yellow vein virus, the causal agent of rhizomania, whereas the soil of the other three locations was virus free. This design would enable the detection marker $\times$ rhizomania interactions. However, the clustering approach for SC and BY did not reveal a subgrouping of the locations with respect to their infestation with rhizomania (Fig. 1). Therefore, we did not further examine the possibility of detecting marker $\times$ rhizomania interactions.

Conclusions

We identified four markers associated with SC, BY, or both. This observation indicates that association mapping can be successfully applied in a sugar beet breeding context for detection of marker-phenotype associations. Furthermore, we conclude from our results that multivariate association mapping is a promising tool for discriminating between pleiotropy and linkage as reasons for co-localization of marker-phenotype associations for different traits with a high mapping resolution.

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