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Genetic dissection of the relationship between carbon metabolism and early growth in maize, with emphasis on key-enzyme loci

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Abstract

The determinism of carbon metabolism traits during early growth in maize has been investigated using a marker-based quantitative genetics approach. In addition to growth traits, concentration of carbohydrates and activity of four key enzymes of their metabolism (sucrose phosphate synthase, ADP-glucose pyrophosphorylase, invertases and sucrose synthase) have been measured in leaves of individuals of a recombinant inbred line population. Using more than 100 RFLP markers, quantitative trait loci (QTLs) were mapped for each biochemical and developmental trait. Causal relationships, suggested by previous physiological studies, were reinforced by common locations of QTLs for different traits. Thus, the strong correlation between growth rate and invertase activity, which may reflect sink organ strength, could be explained to a large extent by a single region of chromosome 8. Moreover, some of the structural genes of the enzymes mapped to regions with QTLs affecting the activity of the encoded enzyme and/or concentration of its product, and sometimes growth traits. These results emphasize the possible role of the polymorphism of key-enzyme genes in physiological processes, and hence in maize growth.

Introduction

With the availability of molecular markers, the chromosome location of genes involved in quantitative trait variation (quantitative trait loci, QTLs), and their genetic effects (additivity, dominance), can be determined, shedding light on the bases of the genetic correlations [34]. This methodology has been efficient in maize for analysing yield and related traits [12, 44] or adaptative traits [33]. QTLs may be tentatively considered as the same genes as those responsible for qualitative mutations, but with minor phenotypic effects [28, 38]. For instance, similar location of QTLs for plant height and dwarf genes has been documented [2, 17]. When genes of known function are cloned, possible consequences of their polymorphism on the variability of certain phenotypical traits can be examined. In maize, a QTL for grain starch and protein content mapped in the region of a gene which encodes the major enzyme of starch synthesis in endosperm [13]. In human

genetics, this 'candidate gene' approach has been efficiently used for the detection of a gene controlling hypertension, a complex disease resulting from multiple genetic and environmental determinants [23]. On the other hand, a search for QTLs controlling biochemical or metabolic parameters may constitute a strategy for analysing the physiological basis of macroscopic trait variation. Whenever QTLs common to the two levels are detected, a causal relationship may be hypothesized, which can be tested by further experiments.

In higher plants, growth and dry matter accumulation are mainly dependent on carbon uptake through photosynthesis and subsequent carbon partitioning between starch and sucrose synthesis. Certain enzymes have been shown to play key roles in carbon metabolism, among which are ADP glucose pyrophosphorylase (AGPase, EC 2.7.7.27) for starch synthesis [35], and sucrose phosphate synthase (SPS, EC 2.3.1.14) for sucrose synthesis [22]. In non-photosynthetic tissues, such as the basal parts of growing leaves, growth is dependent upon the retrieval of sucrose transported through phloem. Two enzymes are responsible for sucrose hydrolysis at this stage, viz. sucrose synthase (SuS, EC 2.4.1.13), and invertase (INV, EC 3.2.1.26). These two enzymes produce hexoses and or nucleotide hexoses which are further used for cell division and growth. The importance of both enzymes have been mainly described during grain development [16], but the role of sucrose synthase activity in the growing part of the leaves has also been shown in maize [32].

We have applied the QTL detection methodology to a population of recombinant inbred lines in maize, in order to dissect the genetic determinism of carbon-enzyme activities and carbohydrate concentrations, and to analyse the extent to which this polymorphism may be involved in the variability of early growth. Moreover, we used the clones of the structural genes of three of the four enzymes studied (SPS, SuS and AGPase) as RFLP probes to search for possible co-locations with relevant QTLs. By dissecting the genetic variability at these three levels, we could obtain insight into the relationship between carbon metabolism and early growth traits, and into the influence of a polymorphism at the key-enzyme loci on these traits.

Materials and methods

Plant material

A population of recombinant inbred lines (RILs) was derived from the cross between an early European flint line ('F2') and a North American line (coded 'Io'), following a single seed descent procedure to F_6 generation, without selection. 'Io' is a private line, obtained from the narrow-genetic-base Iodent group. 145 RILs were retained to construct the RFLP map. Early growth and physiological measures were performed on a sample of 65 RILs, grown in a greenhouse in two sets. The first set was composed of 20 lines, of 6 individuals each, the second of 45 lines, of 3 individuals each. In each set the parental line 'F2' and the F_1 hybrid were measured as controls; 'Io' was measured only in the first set. The seeds were planted in vermiculite in individual pots, in completely randomized designs. Minimal temperature was 25 °C at day and 15 °C at night, relative humidity was 50–60%, and natural light was supplemented 16 h a day with fluorescent lamps (300 μmol quanta m^{-2} s^{-1}). Nutrient solution was supplied [39]. Measures and sampling were performed when the ligule of the third leaf was visible. The measured traits were the number of days from sowing to mature 3rd leaf, the 3rd leaf length and width, the height from the collar to the 3rd leaf ligule, and the dry matter weight of shoot.

Physiological measurements

Three leaf strips (about 30 mg) were cut off in the medium part of the third expanded leaf. The activities of SPS and AGPase, and sucrose (SUC3) and reducing carbohydrate (RED3) concentrations were measured in these samples. A sample of 20–80 mg of the basal part of the fourth growing leaf was removed at the same time for mea-

suring SuS and INV activities. In addition, in the second set (45 RILs), sucrose (SUC4) and reducing carbohydrate (RED4) concentrations were measured in the fourth leaf extract. SPS activities were determined spectrophotometrically by a method derived from Huber [20]. After grinding 20 to 30 mg of lamina at −196 °C in an Eppendorf tube, the leaf powder was warmed to 4 °C with 500 μl extraction buffer (50 mM Hepes-NaOH pH 7.5, 5 mM $MgCl_2$, 2.6 mM dithiothreitol, 1 mM EDTA, 0.6% bovine serum albumin, 0.02% Triton X100). The homogenate was centrifuged for 3 min at 12000 × *g*. The supernatant was desalted by centrifugation on Sephadex G25 columns equilibrated with extraction buffer without Triton X100 [19]. 50 μl of desalted extract was added to a reaction mixture containing 3 mM fructose-6-phosphate, 12 mM glucose-6-phosphate, 6 mM UDP-glucose, 10 mM Pi (limiting substrate condition). The reaction (final volume 70 μl) was incubated for 10 min at 30 °C and stopped by adding 50 μl 5 M NaOH. The hexoses were destroyed by immersing the tubes in boiling water for 10 min. The sucrose content was revealed with 0.15% anthrone reagent in 13.5 M H_2SO_4 for 20 min at 40 °C [15]. The concentration of the reaction product was measured spectrophotometrically at 620 nm by comparison with sucrose standards. AGPase activities were measured on the same extract as SPS, before desalting operation, by an enzymatic method [40]. Invertase and sucrose synthase activities were obtained by grinding 30 to 80 mg of sheath at −196 °C. The leaf powder was warmed to 4 °C with 500 μl extraction buffer (50 mM Hepes-NaOH pH 7.0, 10 mM $MgCl_2$, 2.6 mM dithiothreitol, 1 mM EDTA, 10% ethyleneglycol, 0.02% Triton X100). The homogenate was centrifuged and the supernatant was desalted as for SPS activity. Sucrose synthase activity was measured by an assay adapted from Huber and Akazawa [21]. The reaction was performed at 30 °C with 100 μl extract by adding 900 μl of medium (50 mM Hepes-NaOH pH 7.0, 2 mM $MgCl_2$, 2.6 mM dithiothreitol, 1 mM EDTA) containing 100 mM sucrose, 1 mM ATP, 0.4 mM NAD, 4 units hexokinase, 3.5 units phosphoglucoisomerase, 2 units glucose-6-phosphate (G6P) dehydrogenase. Neutral invertase activity was measured from control slope of absorbance change at 340 nm and sucrose synthase activity after addition of 1 mM UDP. Acid invertase (INV) activity was determined at 30 °C with 25 μl extract, by adding 35 μl of medium (0.2 M acetate buffer pH 4.8) containing 100 mM sucrose. Activity was stopped by adding 50 μl 0.5 M phosphate buffer (pH 7.0). The tubes were immediately immersed in boiling water for 3 min. The glucose and fructose derived from sucrose hydrolysis were determined by adding 700 μl of medium (50 mM Hepes-NaOH pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA) containing 1 mM ATP, 0.4 mM NAD, 4 units hexokinase, 3.5 units phosphoglucoisomerase, 2 units G6P dehydrogenase [4]. Enzyme activities are all expressed in nmole s^{-1} mg^{-1} fresh weight. Carbohydrate (sucrose and hexoses) concentrations were determined with an enzymatic method and expressed in mg g^{-1} [4].

RFLP procedure

The RFLP genotyping of recombinant inbred lines was performed with classical procedures [11]. DNA extractions were performed on bulks of 10 F_6 plants, each bulk representing one F_5 line. The RFLP probes used are 60 genomic probes, kindly provided to us by E. Coe and D. Hoisington, 2 anonymous cDNAs [25], 8 cDNAs whose sequences have homology with genes of known function [25] and 19 clones of genes of known function [6a]. The sugar metabolism genes used as probes were a SPS cDNA (kindly provided by R. Ohsugi), three AGPase cDNAs (*Sh2* and *Bt2* kindly provided by L. C. Hannah, and *L2* by J.L. Prioul), and two SuS cDNAs (*Sh1* provided by L. C. Hannah and *Css1* by A. Lecharny).

Statistical analyses

The RFLP map was constructed using the MAPMAKER/EXP V3.0 software [27]. Linkage

groups were first established with LOD threshold of 3.0 and recombination fraction of 0.3. The order was then estimated using a LOD threshold of 2.0. Some linkage groups separated by long distances were linked together using information obtained from the integrated map constructed using two additional populations [6a]. Distances were expressed in Kosambi cM.

Within each set of RILs, the differences between lines were tested by one-way ANOVAs. Heritabilities were estimated as $\sigma_g^2/(\sigma_g^2 + 1/_n\sigma_e^2)$ where σ_g^2 and σ_e^2 are the estimated genetic and residual variances, and n the number of individuals per line.

As the comparison of the means of the controls of the two sets revealed some differences, we did not use the rough line values, but the differences between line and control values. Marker-trait associations were tested using one-way ANOVAs. QTLs were detected in two steps: chromosomal regions where significant associations between marker and trait were first detected using a $P = 0.01$ threshold. Then a less stringent threshold ($P = 0.05$) was accepted within these regions, to reveal possible low-effect QTLs accounting for correlations between traits. QTL effects were estimated as the percentage of phenotypic variation explained by the QTL (R^2), computed as the ratio of marker to the total sum of squares. Epistasis was tested by two-way ANOVAs with interaction for all pairs of markers.

Results

Genetic variation, RFLP map and marker-trait associations

Table 1 shows the mean values of parental lines and the heritabilities estimated in the 2 RIL sets for growth and physiological traits. The population of recombinant lines showed extensive variation, since significant differences were found between lines for every trait. RIL values out of the range of the parents and F1 hybrid were frequently observed (data not shown). The RFLP map, composed of 109 loci, covered 1337 cM, which represented one marker every 13 cM on average (Fig. 1). At the $P = 0.01$ threshold level, 2.8% of the marker-trait associations were significant, with every trait involved. As some significant loci were linked, 13 chromosomal regions (noted *Qi* with *i* for the chromosome number) were defined involving one to seven loci (Fig. 1), and the R^2 value retained for the region was the highest value observed. Two unlinked regions (denoted A and B) were defined on chromosomes 1, 5, 8 and 10. On chromosome 2, two regions, 30 cM apart,

Table 1. Parental mean values in the 2 sets and heritabilities (h) estimated among the recombinant inbred lines.

	First set (20 RILs)				Second set (45 RILs)		
	'Io'	'F2'	F1	*h*	'F2'	F1	*h*
Morphological traits							
Day number to 3rd leaf	19	19	16	*0.41*	17	15.3	*0.60*
3rd leaf length (mm)	268	326	325	*0.84*	323.3	331.3	*0.86*
3rd leaf width (mm)	13	15	16	*0.79*	11.7	13.7	*0.92*
Plant height (mm)	93	112	116	*0.62*	120	141.3	*0.79*
Physiological traits							
SPS activity	24	28	33	*0.81*	17.8	18.1	*0.41*
AGPase activity	36	37	37	*0.59*	25.5	33.5	*0.35*
INV activity	45	44	33	*0.31*	22.7	25.4	*0.33*
SUS activity	47	35	40	*0.56*	23.87	18.4	*0.47*
Reducing sugars in 3rd leaf	0.7	2.8	1.1	*0.83*	2.83	1.1	*0.60*
Sucrose in 3rd leaf	3.3	4.8	4.3	*0.35*	4.23	3.5	*0.46*

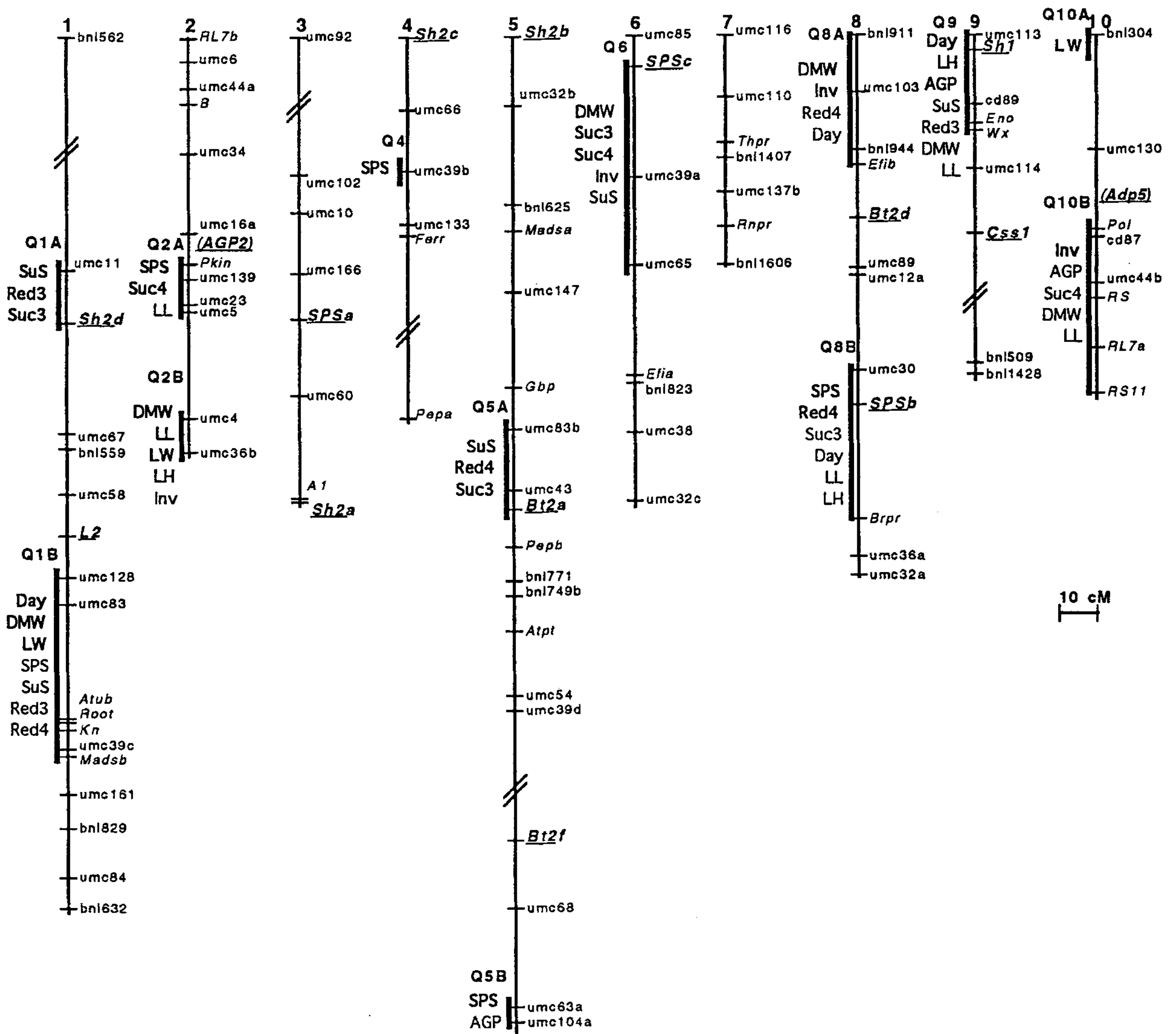

Fig. 1. RFLP map based on a population of 145 recombinant inbred lines from the cross 'Io' × 'F2', constructed with umc (University of Missouri Columbia) and bnl (Brookhaven National Laboratory) genomic clones and cDNA clones with unknown function (cd). Probes corresponding to known function genes are indicated in italics (*A1*, *B* and *RS* are involved in anthocyanin metabolism, *Atub* encodes for α-tubulin, *Ferr* for ferritine, *Kn* for a transcription factor, *Pep* for the phosphoenolpyruvate carboxylase, *Pkin* for a protein kinase, *Pol* for a pollen-specific protein, *RL7*, *RS11* for ribosomal proteins, *Root* for a root specific protein and *Wx* for starch glucosyl transferase). Probes corresponding to expressed sequence tags are *Atpt*, *Brpr*, *Efi*, *Eno*, *Gbp*, *Mads*, *Rnpr* and *Thpr* [24]. When more than one locus corresponded to a probe, a letter is added to the probe name. Loci revealed by hybridization with probes of AGPase (*Bt2*, *Sh2*, *L2*), SPS and SuS (*Css1* and *Sh1*) are underlined. Location of the two loci *ADP5* and *AGP2*, deduced from mapping of other populations are indicated in parentheses. Double bars on chromosomes, when distances are higher than 30 cM, indicate that linkage was deduced from other mapping data obtained in our laboratory on related populations. Distances were calculated in Kosambi cM. Chromosomal regions where QTLs were detected are indicated by vertical lines, with the region name (Qi), to the left of chromosomes, and the traits involved (LW, leaf width; LH, plant height; LL, leaf length; DMW, dry matter weight; DAY, day number) in bold type when significant associations were detected at the $P = 0.01$ threshold.

were defined because the effects on leaf length at markers umc4 and *Pkin* had opposite allele effects, suggesting the existence of at least two linked QTLs. Nine regions had QTLs for more than one trait, among which Q1B and Q2B were specific for growth traits, Q1A, Q2A, Q5A and Q8B for physiological traits, while Q6, Q8A and Q9 were associated with both (Table 2).

QTLs for physiological traits and location of the carbon metabolism genes

Ten of the 13 regions were associated with at least one physiological trait ($P = 0.01$). Four QTLs affected the SPS activity (Q2A, Q4, Q5B and Q8B), one the AGPase activity (Q9), two the INV activity (Q8A and Q10B) and two the SuS activity (Q1A and Q5A). Neutral invertase activity (not shown) was very low but it mapped on QTL similar to INV (acid invertase). One QTL was detected for RED3 and three for SUC3, two for RED4 and two others for SUC4.

AGPase is a protein composed of two distinct subunits. Two mutations affecting AGPase activity in grain allowed cloning and characterizing two structural genes, *Bt2* [1] and *Sh2* [5], both expressed in the endosperm. Two other genes (*AGP1* and *AGP2*) expressed in the embryos were cloned and mapped (Hannah and Giroux, pers. comm.), and a clone homologous to *Bt2*, *L2*, expressed in the leaves has been cloned [37]. According to the maize gene list [9], another locus (*ADP5*) could correspond to a gene coding for AGPase. Nine loci were detected with the probes *Sh2*, *L2* and *Bt2* (four with *Sh2*, one with *L2* and four with *Bt2*). The *L2* gene is on chromosome 1, near umc58. Two probes corresponding to the *Sh2* gene were used (Fig. 2a): the 3′-end probe (1050 bp) mapped as expected on a single locus on chromosome 3 (locus *Sh2a*); the 5′-end of the gene (850 bp) revealed four additional bands corresponding to three additional loci on chromosomes 1 (*Sh2d*), 4 (*Sh2c*) and 5 (*Sh2b*). The *Bt2* probe allowed four loci to be mapped (Fig. 2b), one on the *L2* locus on chromosome 1, two on

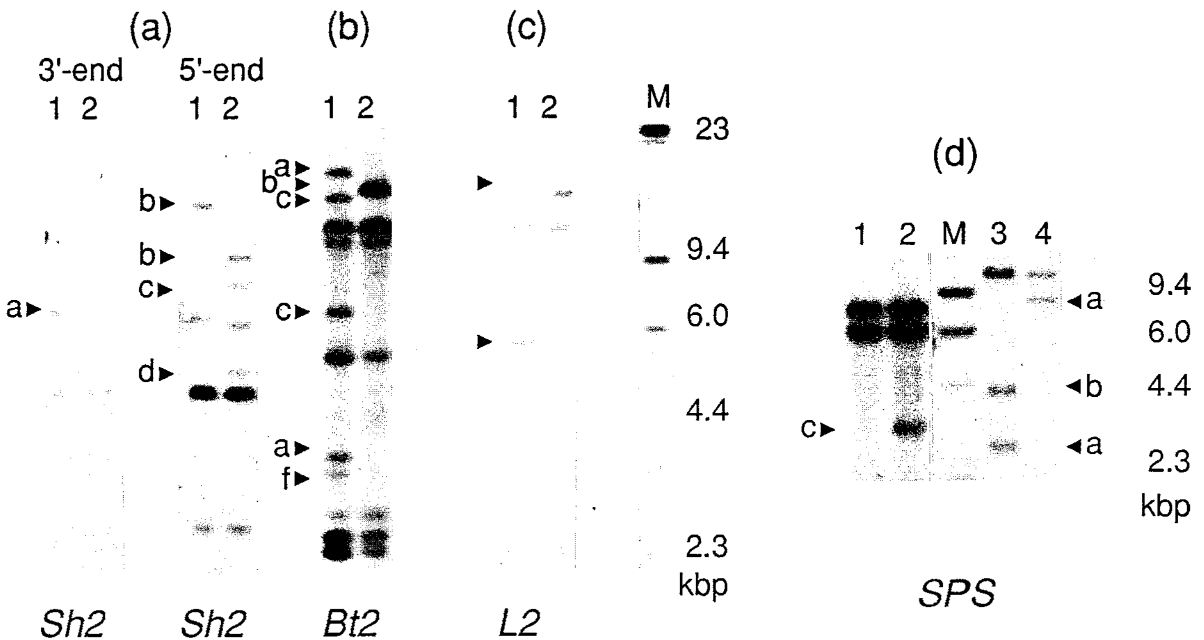

Fig. 2. Autoradiograms resulting from the hybridization of the probes *Sh2* (5′ and 3′ end) (a), *Bt2* (b), *L2* (c) and *SPS* (d) on the genomic DNA of the two parental lines of the RIL population, 'F2' (lanes 1 and 3) and 'Io' (lanes 2 and 4), digested by *Hin*d III (a, b, c) or by *Hin*d III and *Pst* I (d). M: molecular weight marker (Lambda phage DNA digested by *Hin*d III). The arrows indicate the polymorphic bands. Letters refer to the corresponding loci.

Table 2. Variation attributable to QTLs ($R^2 \times 100$) and genotype contributing to the highest values (F: 'F2'; I: 'Io'). Chromosomal regions where significant associations with morphological and/or physiological traits were detected are coded Qi, and characterized by the flanking loci, the number of loci in the region and the size of the region in cM. Carbon metabolism loci mapping in these regions are indicated as candidate loci. Effects detected at the $P = 0.01$ level are underlined.

Chromosomal regions	**Q1A**	**Q1B**	**Q2A**	**Q2B**	**Q4**	**Q5A**	**Q5B**	**Q6**	**Q8A**	**Q8B**	**Q9**	**Q10A**	**Q10B**
flanking loci	umc11	umc128	*Pkin*	umc4	umc39b	umc83b	umc63a	*SPSc*	bnl911	umc30	umc113	bnl304	*Pol*
	Sh2d	umc161	*umc5*	umc36b		umc43	umc104a	umc65	*Efib*	*Brpr*	*Eno*		*RS11*
number of loci (cM)	2 (14)	8 (58)	4 (12)	2 (9)	1	2 (16)	2 (4)	3 (52)	4 (34)	3 (40)	4 (23)	1	6 (42)
candidate loci	*Sh2d*		*AGP2*			*Bt2a*		*SPSc*		*SPSb*	*Sh1*		*ADP5*
Physiological traits													
SPS activity		7(F)	12(F)		23(F)		19(F)			13(F)			
AGPase activity							12(F)				12(1)		10(F)
INV activity				7(I)				7(I)	35(I)				14(F)
SUS activity	12 (I)	7 (I)				12(I)		10(I)			11(I)		
RED3	13(F)	9(F)									9(I)		
SUC3	12(F)					15(I)		18(I)		9 (I)			
RED4 (a)		13(F)				24(I)			17(I)	26 (I)			
SUC4 (a)			22(F)					17(I)					12 (I)
Morphological traits													
Day number to 3rd leaf		14 (I)							8(I)	7 (I)	15(I)		
3rd leaf length			8(F)	25(I)					9(I)	8 (I)	8(I)		7(F)
3rd leaf width		29 (I)		20(I)							10(I)	17(F)	
Plant height				17(I)					13(I)	10 (I)			
Dry matter weight (a)		19 (I)		32(I)				23(I)	20(I)		12(I)		17(F)

(a) Calculated only in 45 lines.

chromosome 5 (*Bt2a* and *Bt2f*) and one on chromosome 8 (*Bt2d*) (Fig. 2b and 2c). None mapped on chromosome 4, where the *bt2* mutation was located [9]. However, due to the partial homology of *Sh2* and *Bt2* [26], *Sh2c* could actually be the *Bt2* locus. The approximate location of *AGP2* and *ADP5* on chromosomes 2 and 10 is indicated in Fig. 1, but this map did not cover the distal part of the chromosome 6 where *AGP1* was mapped. One QTL for AGPase activity was detected on chromosome 10, in the *ADP5* region. Furthermore three of the AGPase loci were located in chromosomal regions where QTLs for other physiological traits were detected, for reducing carbohydrates or sucrose concentrations (Q1A, Q2A, Q5A), for sucrose synthase activity (Q1A and Q5A) and for SPS activity (Q2A). No QTL were detected close to *Sh2* and *Bt2* genes (*Sh2a* and *Sh2c* loci, respectively), but these two genes are known to be expressed only in endosperm. *L2* is expressed in leaves [37] but is linked to no detectable QTL in this progeny.

Three loci were detected with the *SPS* cDNA probe (Fig. 2d), which mapped on chromosomes 3, 6 and 8 (Fig. 1). One of the main QTLs for SPS activity was detected on Q8B, exactly on the *SPSb* locus. A QTL with a great effect on RED4 mapped in the same region. Near the *SPSc* locus, associations were detected for sucrose concentrations (SUC3 and SUC4), INV and SuS activities.

The two structural genes coding for SuS, *Sh1* and *Css1*, mapped as expected on chromosome 9 [29, 42]. An association with SuS activity was found on chromosome 9, near the *Sh1* locus. No QTL were detected close to *Css1*.

Genetic basis for correlations between physiological and morphological traits

Six of the 13 chromosomal regions had QTLs for growth traits (Table 2). Three of these (Q1B, Q2B and Q8A) concerned more than one trait, as expected from their high mutual correlations between them (Table 3). Four QTLs were detected for dry matter weight, and two for day number. Only one out of three regions controlling leaf size affected both leaf length and width.

Significant correlations were found between INV activity and growth traits (Table 3). The two QTLs found for the activity of this enzyme, on Q8A and Q10B, mapped with QTLs for morphological traits, the parent conferring the favourable alleles being the same for both traits. Negative correlations were detected between SPS activity and leaf width, and two of the QTLs (Q1B and Q8B) observed for SPS activity were located in regions where morphological QTLs were detected, acting with opposite allele effect. SuS activity, though not significantly correlated with the number of days, shared QTL locations for this trait on Q1B and Q8A. Other regions shared QTLs for both morphological and physiological traits. For example, two of the three regions where QTLs were found for AGPase activity (Q9 and Q10B) showed QTLs for growth traits (at the $P = 0.05$ threshold level), the two regions showing the same 'high' allele for AGPase activity and morphological traits. RED3 was not correlated with any growth trait, though it shared common QTL locations with morphological traits, the 'high' alleles being the same in one case, and being opposite in the other case. SUC3 and number of days also shared a common QTL location (Q8B).

Epistasis

When considering all pairs of markers, the number of significant interactions was not different from what is expected by chance. Nevertheless, this number often increased when tested only for pairs of markers with at least one significant principal effect on the trait (Table 4). The case of the umc166 locus can be mentioned. This locus, where no QTL was detected, showed a highly significant ($P < 10^{-5}$) interaction with umc104a (on chromosome 5, linked to a QTL for SPS activity) for INV activity (Fig. 3) and day number. It is interesting to note that the significant interactions detected concerned at least one of the 13 candidate gene loci, and this, more frequently than expected by chance, indicating a

Table 3. Phenotypic correlations estimated in the 65 RILs.

	Day number to 3rd leaf	3rd leaf length	3rd leaf width	Plant height	Dry matter weight	SPS activity	AGPase activity	INV activity	SUS activity	Carbohydrate concentrations			
										RED3	SUC3	RED4 (a)	SUC4 (a)
Day number to 3rd leaf	1	0.36**	0.33**	0.45***	0.44**	−0.01	−0.16	0.41***	0.22	−0.07	0.31*	0.00	−0.08
3rd leaf length		1	0.51***	0.80***	0.92***	−0.03	0.07	0.31*	0.10	−0.05	0.20	0.12	0.18
3rd leaf width			1	0.34**	0.77**	−0.26*	−0.15	0.30*	0.23	−0.08	0.21	0.17	0.02
Plant height				1	0.82***	−0.14	−0.06	0.27*	0.03	−0.12	0.09	0.09	−0.04
Dry matter weight (a)					1	−0.06	0.18	0.42**	0.18	−0.04	0.13	0.21	0.04
SPS activity						1	0.38**	0.02	−0.06	−0.05	0.02	0.03	0.11
AGPase activity							1	−0.24	−0.05	−0.18	−0.20	−0.21	0.19
INV activity								1	0.36**	0.10	0.32*	0.35*	−0.21
SUS activity									1	−0.02	0.15	0.00	0.04
RED3										1	0.45***	0.17	−0.02
SUC3											1	0.32*	0.04
RED4 (a)												1	0.20
SUC4 (a)													1

(a) Calculated only in the second set of 45 lines. * $P<0.05$; ** $P<0.01$; *** $P\leq 0.001$.

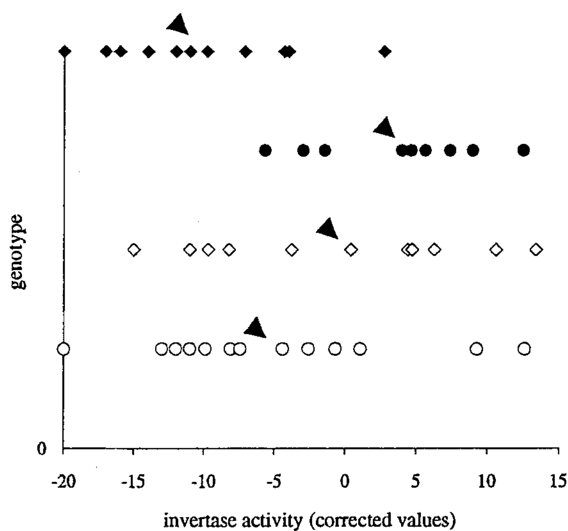

Fig. 3. Distribution of the invertase activity in the 65 RILs depending on the interaction between their genotype at the umc104a and umc166 loci. Open or closed circles correspond to 'F2' genotype at umc104a locus associated, at umc166 locus, with either 'F2' or 'Io' genotypes. Similarly open or closed squares correspond to 'Io' genotype at umc104a locus associated, at umc166 locus, with either 'F2' or 'Io' genotypes. Arrows indicate the average value of each class.

possible role of these genes not only as principal effect but also in interactions with other genes (Table 4). This proportion appeared slightly higher for physiological traits.

Discussion

Detecting QTLs with RILs

The interest of recombinant inbred lines for high-density mapping and QTL detection has been previously reported [6]. The absence of heterozygotes, even though it precludes the estimation of dominance effects at the QTLs, has two advantages: dominant markers are as informative as co-dominant ones, and, due to the increased genetic variance, the power for QTL detection is improved relative to that of F_2 progeny. However, for any recombination fraction r between the marker and the QTL, the RIL populations are less efficient than the other segregating popula-

Table 4. Percentages of the tests for two locus epistasis significant at $P < 0.05$ or $P < 0.01$, compared to the percentages expected by chance. Tests were performed on all the pairs of markers, on pairs involving 1 QTLall markers 2 QTL, or at least one of the 13 carbohydrate metabolism loci (candidate loci). Values higher than two times the expected frequency are underlined.

Probability threshold	Significant tests involving	% expected by chance	Physiological traits				Carbohydrate concentrations				Morphological traits				
			SPS activity	AGPase activity	INV activity	SUS activity	RED3	SUC3	RED4	SUC4	Day number	3rd leaf length	3rd leaf width	3rd leaf heigth	dry matter weight
$P = 0.05$	All marker pairs	5	5	5	5	5	5	5	5	4	6	5	5	6	5
	1 QTL × 1 marker	5	5	7	5	5	7	6	9	5	10	8	6	6	5
	2 QTLs	5	15	5	15	8	15	8	15	5	19	16	14	6	5
$P = 0.01$	All marker pairs	1	0.9	0.8	1.2	0.7	1.3	1.1	1	0.6	1.5	1.5	1	1	1
	1 QTL × 1 marker, or 2 QTLs (a)	1	0.6	1.9	1.0	0	4.5	1.1	3.5	0.7	3.3	4.7	0.7	2	1.7
$P = 0.01$	At least one candidate locus	1	2.6	1.5	2.1	2.5	2.6	2.1	1.8	0.9	2.3	2.1	0.9	3.4	2.2

(a) At the $P = 0.01$ threshold, the number of tests involving 2 QTLs is too low to be mentioned.

tions for QTL detection, because the succession of meioses increases the apparent r value, decreasing the additive value estimated at the marker [10]. Moreover, the moderate size of our sample (65 lines) did not allow low effect QTLs to be detected. For these reasons, the number of QTLs found must be considered as a minimal estimate. The threshold we chose results from a compromise between preserving a sufficient power and limiting the risk of false-positives. It is worth noting that the small size of the progeny does not modify the percentage of false-positive QTLs, which is given by the type I error, regardless of the sample size. With $P = 0.01$, and 100 markers, one false-positive QTL on the average is expected to be found by chance for a given trait. Actually the markers are not independent, so this figure is highly overestimated, and we found several QTLs for most of the traits. The more stringent the threshold, the less numerous are the false-positive QTLs, but the more numerous are the actual QTLs remaining undetected.

The candidate gene approach

In maize, numerous enzymes are encoded by more than one gene, with specific organ, tissue or organelle localization. Furthermore, maize is a C4 plant with specialized cell types in leaves: mesophyll cells where 80% of the sucrose is synthesized, and bundle sheath cells where starch accumulates. Possibly, there are different homologous genes for each cell type, as suggested for pyruvate orthophosphate dikinase [41]. If this is generally true for other genes, it would explain the numerous loci revealed by hybridization with *SPS* as well as *Bt2* and *Sh2*. An additional explanation might be the duplicate origin of the maize genome [18]. *SPSb* and *SPSc*, for example, are located in two regions showing many other homologies.

Among the 15 putative candidate genes, three were found close to a QTL for the activity of the corresponding enzyme: *SPSb* in Q8B, *Sh1* in Q9, and *ADP5* in Q10B. SPS activity in maize is supposed to be conferred by a single gene product [46]. Among the three loci detected for SPS,

SPSb could be the functional one, since it mapped with a QTL affecting SPS activity, the two other loci being either pseudogenes or genes expressed in other cell types or organs. The three QTLs for SPS activity found at Q2A, Q4 and Q5B may reveal variations at various regulatory steps, since the activity of this enzyme is regulated by the protein quantity, substrate availability, activator or inhibitor concentrations, phosphorylation state following diurnal variation, and also hormonal level [22].

Sucrose synthase is a protein encoded by two genes, which are spatially and temporally regulated [7], and these regulations are strongly interrelated [30]. *Css1* seems to be regulated differently than *Sh1*, which is mainly expressed in starch storage cells. In developing leaves, the majority of the protein consists of the isoform encoded by *Css1* gene [15], but a slight expression of *Sh1* is also detected in leaf [32]. A QTL for this activity was still found nearby to the *Sh1* gene, suggesting a control of *Css1* expression by a sequence near or within *Sh1*. This result is in accordance with the epistatic interaction between the two genes detected in mutants [8].

In endosperm, AGPase is a heterotetramer composed of two subunits encoded by the *Sh2* and *Bt2* genes [36]. In leaves, the structure is supposed to be the same, but the polypeptides might be encoded by two other genes, one of them being *L2* [37], which shares strong homology with *Bt2*. The other gene, which should present homology with *Sh2*, has not yet been cloned, but could be one of the loci revealed by homology with the 5′-end of the *Sh2* gene, or the *ADP5* locus located on chromosome 10, close to a QTL for the activity of this enzyme.

A mutant, called miniature (*mn1*), which presents a very reduced invertase activity in kernels, mapped on chromosome 2, near umc34 [31]. No QTL was found in this region for INV activity, but it is not clear whether this mutation corresponds to the structural gene or to a regulatory one. The cloning of two invertase maize cDNA in maize has been reported [47]. Mapping these clones in our population will be of great interest.

Relationships between physiological traits

A QTL common to SPS and AGPase is observed at Q5B. In leaves, starch synthesis takes place in the chloroplast while sucrose synthesis is cytosolic. Sucrose synthesis is dependent on the concentration of metabolites (3-P-glycerate and triose phosphate) formed in the chloroplast during photosynthesis. They must be transported through the chloroplast envelope via the phosphate translocator. The 3-P-glycerate is an activator of AGPase and the inorganic phosphate is a common inhibitor of SPS and AGPase [44]. Thus, any gene controlling these concentrations or transportation might be a QTL for these two activities.

SuS and INV activities are two unrelated mechanisms for sucrose hydrolysis. One common QTL (Q6), also shared with sucrose concentrations (SUC3 and SUC4) could reflect the fact that the activity of both enzymes depends upon their common substrate, sucrose [26]. Common QTLs between sucrose or reducing carbohydrate concentrations and enzyme activities were also detected. Finally, epistatic interactions revealed a complex network between the genes controlling the carbon metabolism in leaves, and a prevalent role of the candidate genes in epistasis, suggesting that they act not only in an additive manner, but also in interactions acting, for example, as limiting factors of another reaction.

Role of enzyme activities in morphological traits

All the morphological traits were highly correlated, and shared numerous QTLs. The observed correlations may be due to linked genes, or to pleiotropic effects of QTLs (dry matter weight is for example a resultant of all the size traits). Physiological and growth QTLs were located on the same regions in 8 of the 13 cases. In some instances a causal relationship can be suspected, as for the strong correlation between INV activity and growth rate. Action of INV activity on node elongation has already been mentioned in oat and barley [24]. SuS activity is also important in growing organs in order to supply cells with

nucleotide sugars useful for wall synthesis. QTLs common to SuS activity, dry matter weight and day number were found at Q1B and Q9. It is interesting to note that, in the latter case, the structural gene *Sh1* mapped in the same region, and could be the causal factor. One QTL common to AGPase and growth traits was also detected (Q9). In leaves, starch is a transient carbon pool which is used late in the day, and increasing the AGPase activity could result in a higher physiological vigor. SPS activity is a key step in carbohydrate metabolism in photosynthetic leaves. This activity has been found to be strongly correlated with the growth rate of maize genotypes [40]. In the cross studied here, a negative correlation was detected between SPS activity and growth, which was also expressed at the QTL level, as two QTLs had opposite effects for growth traits and SPS activity (Q1B and Q8B). At Q2A, nevertheless, 'F2' alleles contributed to longer leaf and higher SPS activity. Thus SPS activity might not be the only growth limiting factor between the RILs studied here.

Conclusion

Considering biochemical parameters as quantitative traits, we were able to map QTLs involved in their control. The oligo or polygenic determinism observed for enzyme activities is consistent with the complexity of their regulation, and with the genetic data from several organisms, including maize, which have shown evidence for diversity within and between species for genes controlling the activity/amount of various enzymes [3, 43]. These results demonstrate that numerous polymorphic factors dispersed throughout the genome are a common feature of enzyme regulation, that might be favourable in some developmental contexts, or in response to environmental changes.

By studying the physiological level, we could obtain insight into the impact of carbon enzyme activities upon growth. Four loci corresponding to carbon-metabolism genes (*SPSb*, *SPSc*, *Sh1* and *ADP5*) were associated with both enzyme activities and morphological traits. Various strategies can be proposed to validate the candidates, such as the study of other genetic backgrounds and greater samples, the analysis of the sequence polymorphism in a range of unrelated genotypes to determine the sequences responsible for the trait variation, and/or the transformation of a genotype with various alleles. Nevertheless, when a gene maps with a QTL for a carbon enzyme activity and/or for carbohydrate concentration, there is a high probability that the locus is the QTL itself.

The availability of a growing number of genes makes this approach more realistic than chromosome walking for the characterization and study of QTLs. In maize, there are around 200 cloned genes or cDNAs. This number is increasing rapidly due to random sequencing [25] and gene tagging experiments. The availability of the QTLs themselves instead of markers of QTLs will make marker-assisted selection more efficient, and will allow rapid PCR tests or transformation to be performed.

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