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## QTL controlling root and shoot traits of maize seedlings under cold stress

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**Abstract** The improvement of early vigour is crucial for the adaptation of maize (*Zea mays* L.) to the climatic conditions of central Europe and the northern Mediterranean, where early sowing is an important strategy for avoiding the effect of summer drought. The objectives of this study were to identify quantitative trait loci (QTL) controlling cold-related traits and to investigate the relationships among them. A set of 168 F<sub>2:4</sub> families of the Lo964 × Lo1016 cross was grown in a sand-vermiculite substrate at 15/13°C (day/night) until the one-leaf stage. Twenty QTL were identified for the four shoot and two seed traits examined. Analysis of root weight and digital measurements of the length and diameter of primary and seminal roots led to the identification of 40 QTL. The operating efficiency of photosystem II ( $\Phi_{PSII}$ ) was related to seedling dry weight at both the phenotypic and genetic level ( $r=0.46$ , two matching loci, respectively) but was not related to root traits. Cluster analysis and QTL association revealed that the different root traits were largely independently inherited and that root lengths and diameters were mostly negatively correlated. The major QTL for root traits

detected in an earlier study in hydroponics were confirmed in this study. The length of the primary lateral roots was negatively associated with the germination index ( $r=-0.38$ , two matching loci). Therefore, we found a large number of independently inherited loci suitable for the improvement of early seedling growth through better seed vigour and/or a higher rate of photosynthesis.

### Introduction

Maize (*Zea mays* L.) cultivars that show early vigour are desirable for enhancing crop establishment in areas in which low temperature retards early seedling growth (Miedema 1982) or where breeding for earliness is a strategy for avoiding the effect of drought at flowering (Mock and McNeil 1979). There are three stages of early plant development: germination, heterotrophic growth and early autotrophic growth. The performance of the genotypes during these stages is referred to as seed vigour (Perry 1987), seedling vigour and early vigour (Revilla et al. 1999), respectively. Depending on the breeding material and the environment, tolerance to cold at all three stages may be controlled by the same set of genes (Mock and Eberhart 1972) or may be inherited independently (Hodges et al. 1997; Mock and Eberhart 1972; Revilla et al. 2000). Ideally, a vigorous genotype will perform well during all these stages (Hodges et al. 1997; Revilla et al. 2000). While seed vigour ensures an uniform germination and plant establishment (Perry 1987), early vigour ensures successful transition to the autotrophic growth stage. Heterotrophic carbohydrate supply can influence plant performance significantly (Revilla et al. 1999) but, in particular under cold stress, the development of a functional photosynthetic apparatus may be of great importance as early as the very early growth phase (Hund 2003).

The critical temperature threshold for maize growth coincides with the soil temperature during the early developmental stages (Richner et al. 1996) and ranges from 10°C to 17°C depending on the trait and cultivar (Blacklow 1972; Bowen 1991; Haldimann et al. 1996).

This paper is dedicated to our friend and colleague Alberto Soldati, who passed away unexpectedly.

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Low temperature affects leaf development (Beauchamp and Lathwell 1966; Stone et al. 1999), photosynthetic efficiency of the seedlings (Fryer et al. 1998; Leipner et al. 1999; Stirling et al. 1991) and root development (Engels 1994; Stamp 1984). Consequently, shoot development at suboptimal soil temperatures may be limited due to a direct effect of temperature on the shoot meristem or to a reduced supply of nutrients through the roots (Engels and Marschner 1990). In general, a highly structured root system is associated with vigorous plant development at the early stages in both the growth chamber (Richner et al. 1997; Stamp 1984) and the field (Richner et al. 1996, 1997).

Detailed descriptions of maize roots are scarce, and the information on quantitative trait loci (QTL) for root traits that is available is mainly for the number (Guingo et al. 1998; Lebreton et al. 1995), weight (Tuberosa et al. 2002b), diameter (Guingo et al. 1998; Tuberosa et al. 2002b) or pulling strength (Landi et al. 2002; Lebreton et al. 1995) of the roots but not for their length. There has been no report of a study that has searched for QTL controlling root morphology under mild-chilling stress, as it occurs commonly in the field. On the other hand, QTL have been mapped for the cold tolerance of the photosynthetic apparatus (Fracheboud et al. 2002). In the population examined in this study, QTL for cold tolerance at germination (E. Frascaroli et al., unpublished data) and for root traits in hydroponics and in the field (Tuberosa et al. 2002b) have already been described. The goal of this study was to identify QTL for vigour-related morphophysiological traits in order to determine the relationship among traits at low temperature and the consistency of root morphology across the QTL experiments.

## Material and methods

### Plant material

A population of 168 maize (*Zea mays* L.) F<sub>2,4</sub> families of a cross of two dent parents (Lo964 × Lo1016) was used for the QTL experiment. The parents were chosen on the basis of their contrasting root morphology (Sanguineti et al. 1998) and their different levels of cold tolerance at germination (E. Frascaroli et al., unpublished data). Lo964 is characterised by a very intensive root system (a dominating primary root) and a high cold tolerance at germination; Lo1016 develops an extensive root system (uniform root types) and its cold tolerance at germination is low. The Lo964 and Lo1016 inbred lines, together with 168 F<sub>2,3</sub> families derived from a corresponding number of randomly chosen F<sub>2</sub> families, were provided by Dr. M. Motto (Experimental Institute for Cereal Crops, Bergamo, Italy). The F<sub>2,4</sub> seeds were produced in the nursery of the Department of Agro-environmental Science and Technology (DiSTA; University of Bologna, Italy) by selfing 20 randomly selected plants for each F<sub>2,3</sub> and bulking an equal amount of seeds for each ear.

### Experimental conditions

Twenty seeds per genotype with a family-specific kernel weight ( $\pm 10\%$ ) were imbibed for 12 h at 16°C on filter paper in 9-mm petri dishes. The seeds were then surface-sterilised with 2.5% NaOCl for

12.5 min, washed three times with tap water and irrigated with 2 ml of captolate solution (54% Captan, 35% Antrachinon, Omnya, Switzerland) at a concentration of 1 ml l<sup>-1</sup>. The seeds were germinated at 25°C; then germination was recorded and the seeds placed into PVC columns (7-cm diameter, 25-cm height) when the primary root length was about 3–10 mm. Three germinated seeds per PVC column were placed 2 cm below the surface of the growth substrate.

The growth substrate was a mixture of quartz sand (particle size: 0.08–0.2 mm) and 5% w/w vermiculite powder (Vermex Pulver E, Vermica AG, Bözen, Switzerland) with a volumetric water content of 25%. The water content was adjusted with a modified Hoagland solution containing 7.0 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.0 mM MgSO<sub>4</sub>, 1.0 mM H<sub>3</sub>PO<sub>4</sub>, 1.5 mM K<sub>2</sub>SO<sub>4</sub>, 0.16 mM FeNa-EDTA, 0.05 mM KCl, 18.0 μM MnSO<sub>4</sub>, 12.0 μM H<sub>3</sub>BO<sub>3</sub>, 1.5 μM ZnSO<sub>4</sub>, 0.6 μM CuSO<sub>4</sub> and 4.2 μM MoO<sub>3</sub>. A pH=7 was obtained by adding H<sub>2</sub>SO<sub>4</sub> to the nutrient solution. The moist substrate was packed into PVC columns to a bulk density of 1.25 g cm<sup>-3</sup> and was covered with a 1.5-cm isolation layer of Perlite (PePe Pflanzen Perlit, Otto Hauenstein Samen, Rafz-Biberist-Landquart-Orbe, Switzerland).

After coleoptile emergence, the PVC columns were placed in a growth chamber (PGW36, Conviron, Winnipeg, Canada). The environmental conditions were set at 12-h-long photo- and thermo-periods, 60/70% (day/night) relative humidity, 15/13°C (day/night) temperatures and 500 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density (PPFD). The temperature of the substrate was recorded at a depth of 5 cm. Plants were grown until the one-leaf (V1) stage, which was defined by a completely visible collar of the first leaf (Ritchie and Hanway 1984).

### Measurements

To detect possible maternal effects, we measured average grain weight on three sub-samples of 50 seeds per genotype. Germination was recorded twice per day during the first 4 days and then daily until the seventh day in three independent replications. A germination index (GI) was calculated using the formula of Smith and Millet (1964)  $GI = [\Sigma (\text{number of seeds germinated on a given day}) \times (\text{number of days after imbibition})] / \text{total number of seeds germinated after 7 days}$ . The percentage of germinated seeds was recorded 7 days after imbibition.

All of the photosynthesis-related measurements were performed at the one-leaf stage half-way between the collar and the leaf tip at leaf temperatures of 17°C. Chlorophyll fluorescence was measured on the first and second leaves with a pulse amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany). The operating efficiency of photosystem II ( $\Phi_{PSII}$ ) was measured on light-adapted leaves (500 μmol m<sup>-2</sup> s<sup>-1</sup>). The maximum quantum efficiency of photosystem II primary photochemistry (Fv/Fm) was determined on dark-adapted leaves. The chlorophyll contents (SPAD values) of the second and third leaves were measured with a Minolta SPAD-502 chlorophyll meter (Minolta, Ramsey, N.J.). The leaf area of the fully developed first leaf and successive leaves was measured with a leaf-area meter (Li-Cor 3100, Lincoln, Neb.).

The root system was removed from the growth columns, washed under pressurised tap water and separated into different root types. The root types (primary, seminal and crown roots) are named according to the nomenclature used by Feix et al. (2000). The main axes of the different root types are termed “axile” according to Cahn et al. (1989), while those roots arising from the axile roots are termed “lateral.” The roots were stained with 75 mg l<sup>-1</sup> fuchsin dye (Pararosaniline P-1528; Sigma, St Louis, Mo.) for 12 h at 4°C and rinsed under running tap water. The lateral roots were cut from the main axis, and both the axile and lateral roots were distributed separately on a glass tray in a thin layer of water. The tray was placed on a flatbed scanner (Scanjet 4c, Hewlett Packard, Calif.) with a toplight adapter (Scanjet 4c/T) to obtain 8-bit greyscale images (resolution: 600×600 dpi) with a uniform background. The scanned root images were analysed with the computer programme RD (ROOT DETECTOR, ETH Zurich, Switzerland) (Walter and Bürgi

1996), which enables sorting of the measured root lengths into user-defined diameter classes. To obtain the length per diameter-class distribution (LDD), we used a diameter-class spacing of two pixel (84.66  $\mu\text{m}$ ). Following calibration with a binocular microscope (bino.) measurements ( $\text{Øbino.} = 1.28 \times \text{ØRD} - 51.61$ ,  $r^2 = 0.95$ ), an effective diameter-class spacing of 108.54  $\mu\text{m}$  was obtained, which represents the highest resolution of the scanning system. The dry matter was separately determined after drying at 65°C for 42 h for the stem, leaves and the axile and lateral roots of each of the three different root types.

### Statistical analyses

The genotypes (two parental inbreds and 168  $F_{2:4}$  families) were arranged in a randomised complete-block design with two replications in time (experiments) and two blocks per experiment. Experimental units (PVC columns), each containing three plants, were arranged as a central and a border block according to the light intensity in the growth chamber. Each block contained one experimental unit per  $F_{2:4}$  family and four paired experimental units of the parental inbred lines.

The PROC MIXED procedure of SAS ver. 8.02 (SAS Institute, 1999–2001, Cary, N.C.) was used to calculate the adjusted means and to estimate the genetic coefficient of variation ( $CV_G$ ) and the broad-sense heritability ( $h^2$ ) of the families.  $CV_G$  is defined as  $\sigma_G / \text{mean}_G \times 100$  (%), where  $\sigma_G$  is the restricted maximum likelihood (REML) estimate of the standard deviation of the factor genotype ( $F_{2:4}$  families), and  $\text{mean}_G$  is its mean value.  $h^2$  was calculated as  $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2/2 + \sigma^2/4) \times 100$  (%), where  $\sigma_{GE}^2$  and  $\sigma^2$  are the REML estimates of variance for ‘genotype  $\times$  experiment’ and error, respectively. Simple Pearson correlation coefficients ( $r$ ) were calculated between the traits using the adjusted means of the  $F_{2:4}$  families. The significance of the correlation coefficient at  $P \leq 0.05$ , 0.01, and 0.001 is indicated as \*, \*\* and \*\*\*, respectively.

A hierarchical cluster analysis of the correlation distance matrix among traits was performed using the ‘hclust’ function of the ‘mva’ package of R 1.6.2 (Ihaka and Gentleman 1996). According to the recommendations of Backhaus et al. (1994) two agglomeration methods were used successively; first, the single agglomeration method to detect outliers, then the Ward’s minimum variance method for the final model.

### QTL analyses

QTL analysis was performed using a linkage map with 135 markers, published by Tuberosa et al. (2002b). Composite interval mapping (CIM) was used to identify the QTL and to estimate their effects (Jansen and Stam 1994; Zeng 1994). All calculations were performed with the software developed by Zeng (1994), which is based on QTL mapping by the mixed models and the maximum

likelihood approach (Haley and Knott 1992) in combination with the use of selected markers as cofactors. A set of three successive models was used to select cofactors and increase the power of CIM: (1) simple interval mapping for the selection of cofactors, (2) CIM only with unlinked markers as cofactors; (3) CIM with selected markers as cofactors and two markers flanking the tested interval but at least 30 cM from the interval. The markers closest to the maximum of the likelihood profile were chosen as cofactors when a LOD threshold of 3 was exceeded. A QTL was declared as significant when a critical LOD threshold of 3.6 was exceeded. For a joint analysis of an  $F_2$  population in two experiments, this threshold is equivalent to a comparison-wise Type-I error probability ( $\alpha$ ) of 0.005 in a  $\chi^2$  distribution with five degrees of freedom ( $df$ ). For the seed traits, QTL were estimated on the basis of the means of three replications, and a corresponding LOD threshold of 2.8 for  $\alpha = 0.005$  was used. Assuming that each chromosome arm segregates independently, the corresponding experiment-wise Type-I error probability ( $\alpha'$ ) is approximately 0.1. A joint analysis of the phenotypic data of the two experiments enabled the evaluation of the QTL-by-experiment (Q $\times$ E) interaction (Jiang and Zeng 1995). The factor ‘experiment’ accounts for the variability in time and space (for technical reasons different growth chambers were used). A LOD threshold of 1 for a significant Q $\times$ E interaction was based on the Type-I error rate for a single locus of an  $F_2$  plant with  $df = 2$ . Multiple regressions were used to evaluate the actual additive effects of QTL and the total percentage of phenotypic variation accounted for by the identified QTL. Because phenotypic evaluation was made on  $F_{2:4}$  plants, only the additive effect of the QTL are reported. QTL positions are described as chromosome number followed by the position of the QTL peak in parenthesis. Distance is given in centiMorgans (cM). In the event of several overlapping peaks, the position of the first and the last peak is given in parenthesis (e.g. chromosome 1 (34–39) = chromosome 1, position 34–39 cM).

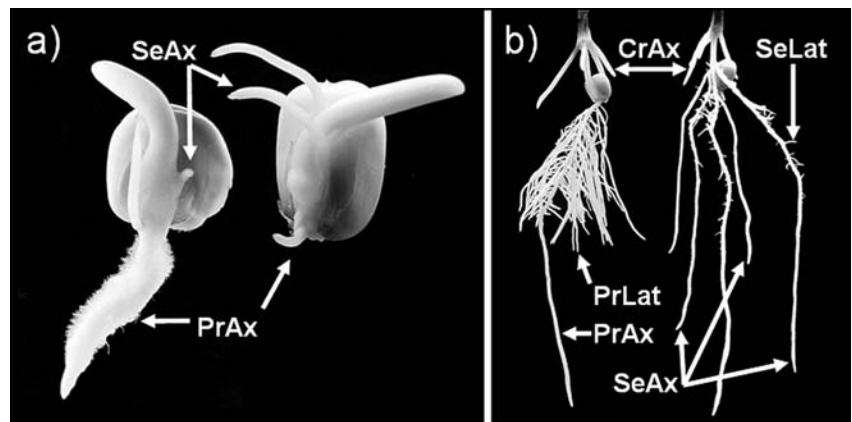
## Results

### Root traits

#### Image analyses

Observations on the root systems of Lo964 and Lo1016 revealed differences that were visually distinct as early as germination (Fig. 1a). Lo964 germinates with a strong primary root that has abundant root hairs, while Lo1016 germinates with a weaker primary root that is soon followed by at least two seminal roots of the same size as the primary root. At the one-leaf stage the parents still differ considerably with respect to root morphological

**Fig. 1** Root system of maize (*Zea mays* L.) dent inbred lines Lo964 (left) and Lo1016 (right) at germination at 25°C (a) and at the one-leaf stage after 2 weeks of growth at 15/13°C (day/night) in a sand-vermiculite substrate (b). CrAx Crown axile roots, PrAx primary axile root, PrLat primary lateral roots, SeAx seminal axile roots, SeLat seminal lateral roots



organisation. Lo964 is a genotype in which the primary root has a strong main axis and a high number of long, thick lateral roots. Lo1016 is characterized by a primary root that is equal in size to its seminal roots (Fig. 1b).

The digital image analysis confirms the visual characterisation of the parents. Figure 2 shows the LDD of the parents and the family means. The lengths per diameter class classification of the primary and seminal roots shows a bimodal frequency distribution due to the differences in the diameter of the main and lateral roots. Distinct peaks mark the lateral roots (left) and main roots (right) of all the genotypes. Figure 2a shows that the diameter distribution of the primary roots of Lo1016 reaches a first maximum at  $0.33 \pm 0.05$  mm, which represents the primary lateral roots (PrLat), and a second one at  $1.41 \pm 0.05$  mm, which represents the primary axile roots (PrAx). Compared to Lo1016, the LDD curve of Lo964 is shifted towards higher classes, reaching a peak at  $0.44 \pm 0.05$  mm for PrLat and at  $1.85 \pm 0.05$  mm for PrAx. The corresponding LDD curve of the  $F_{2:4}$  families is intermediate between those of the parents. The peaks of the seminal lateral roots (SeLat) of Lo1016 and of the  $F_{2:4}$  families are both located at  $0.33 \pm 0.05$  mm, whereas Lo964 had developed hardly any SeLat by the time of harvest (Fig. 2b). The LDD peaks of the seminal axile root (SeAx) of the parents and the families are all at the position  $1.1 \pm 0.05$  mm. All of the peaks are symmetric, which enables calculation of mean root diameters from the LDD data.

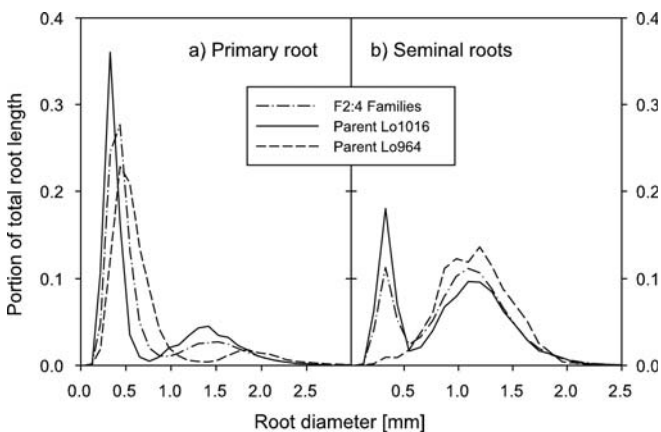
### Phenotypic analyses

The restricted maximum likelihood analysis revealed that differences in the root traits between the parents were significant only for the components of the primary root, namely PrAx length, PrLat length and PrAx diameter (Table 1). While the lateral roots of the parents made up a large proportion of the overall root length (35% for Lo1016 and 74% for Lo964), they contributed little to the

dry weight (DW) of the roots (6% for Lo1016 and 19% for Lo964). This discrepancy underlines the necessity to measure structure rather than DW for a better understanding of root morphology. PrLat was the prevalent root type of the parent Lo964, representing 73% of the overall root length, while SeAx was the predominant root type of Lo1016, representing 49% of the overall root length. The highest  $CV_G$  among the root traits of the  $F_{2:4}$  families was for the length of the lateral roots; the lowest for root diameters and number of axile roots. A transgressive segregation, where the  $F_{2:4}$  families showed values outside the range of the parental genotypes, was observed for all of the root traits. The root counts were unreliable for distinguishing genotypes since heritability was the lowest for these traits, while SeAx diameter and PrAx length were most reliable for distinguishing genotypes on the basis of their high heritability.

### QTL analyses

QTL analysis was performed on the components of the embryonic root system (primary and seminal roots) only since these represented 94% of the length of the seedling root system. Table 2 lists the main parameters of the 40 QTL detected ( $LOD > 3.6$ ), six for root weight, 13 for root diameter, 16 for root length and five for the SeAx count. When we considered as overlapping those QTL the peaks of which were within a region of 10 cM, the QTL could be grouped into 26 chromosomal regions, nine of which influenced at least two of the root traits investigated. The highest joint LOD scores were observed on chromosome 4 (10) for SeLat diameter and on chromosome 6 (20) for SeLat length ( $LOD$  of 11.1 and 7.9, respectively); both QTL showed high LOD scores for the  $Q \times E$  interaction. QTL with LOD scores above five and a  $Q \times E$  interaction below the threshold of one were observed on chromosome 1 at 44, 70 and 81 cM as well as on chromosome 3 at 20 cM and 41 cM and chromosome 5 at 113 cM. Depending on the experiment and the trait, 8.6–42.3% of the phenotypic variability among the families was explained (Table 2). A negative sign of the additivity indicates that the trait-increasing alleles were contributed by Lo964. With respect to the sign of the additivity, similar numbers of increasing alleles were distributed among the parents, with the only exceptions being length of both the SeLat (three of four positive alleles from Lo1016) and the SeAx (all positive alleles from Lo1016) as well as the diameter of the PrLat (three of four positive alleles from Lo964). The sum of the estimated additive effects, compared to half the difference between the parental lines, was lower for the SeLat length (2.35 vs. 8.0 cm), the SeAx diameter ( $-0.064$  vs.  $-0.015$  mm) and the SeAx count (0.39 vs. 1.65) but was higher for the PrLat length ( $-1.23$  cm vs.  $-29.55$  cm), the diameter of PrAx ( $-0.055$  vs.  $-0.240$  mm) and the diameter SeLat (0.005 vs.  $-0.065$  mm). This indicates that some of the factors responsible for the differences between the parental lines were not detected.



**Fig. 2** Length per diameter-class distribution of primary (a) and seminal (b) roots of the two parental maize inbred lines (Lo964 and Lo1016) and their  $F_{2:4}$  offspring (average of 168 families) at the one-leaf stage. Diameter classes are spaced at regular intervals of two pixels, which correspond to a resolution of 0.1085 mm

**Table 1** Summary statistics of the root morphological traits of the two parental inbred lines and their F<sub>2:4</sub> segregating offspring. Plants were grown in two independent experiments with eight replicates for the parents and two for the F<sub>2:4</sub> families

Root type	Trait	Parental means		F <sub>2:4</sub> families				CV <sub>G</sub> (%) <sup>a</sup>	h <sup>2</sup> (%) <sup>b</sup>
		Lo964	Lo1016	Maximum	Minimum	Mean			
Overall									
Axile (Ax)	Dry weight (mg)	25.7	24.1	43.2	10.9	24.0	***d	17	63
Lateral (Lat)	Dry weight (mg)	6.2	1.4	19.5	0.0	2.7	***	36	57
Axile	Length (cm)	25.7	53.4	87.3	27.9	48.8	***	15	56
Lateral	Length (cm)	71.8	28.8	105.4	0	45.4	***	40	67
Primary (Pr)									
Axile (PrAx)	Length (cm)	9.1	7.38	17.5	0	9.9	***	20	72
	Diameter <sup>c</sup> (mm)	1.87	1.39	1.90	1.07	1.51	***	7	63
Lateral (PrLat)	Length (cm)	71.4	12.3	96.1	0	35.9	***	43	65
	Diameter (mm)	0.46	0.35	0.61	0.34	0.43	***	7	70
Seminal (Se)									
Axile (SeAx)	Count	1.9	5.2	8.4	2.2	4.6	*	12	30
	Length (cm)	10.1	40.4	65.9	13.8	33.7	***	19	55
	Diameter (mm)	1.24	1.21	1.52	0.87	1.18	***	9	78
Lateral (SeLat)	Length (cm)	0.4	16.4	44.3	0.1	9.5	***	47	55
	Diameter (mm)	0.46	0.33	0.47	0.26	0.36	***	6	51
Crown (Cr)									
Axile (CrAx)	Count	3.2	2.9	4.2	1.7	3.0	*	10	34
	Length (cm)	6.4	5.7	11.1	1.2	5.4	***	24	54

\*, \*\*\*, Comparison of means significant at  $P \leq 0.05$  and  $0.001$ , respectively. NS, Not significant

<sup>a</sup>The genetic coefficient of variation was calculated from the REML estimate of variance

<sup>b</sup> $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2 / 2 + \sigma^2 / 4)$ , where  $\sigma_G^2$ ,  $\sigma_{GE}^2$ , and  $\sigma^2$  are the REML estimates of variance for the genotype, genotype  $\times$  experiment, and error, respectively

<sup>c</sup>Comparison of parental inbreds

<sup>d</sup>Comparison of F<sub>2:4</sub> families

<sup>e</sup>Mean diameter was calculated from the length per diameter-class distribution

**Table 2** Putative QTL for root traits (LOD>3.6) of 168 F<sub>2:4</sub> families of the Lo 964 × Lo 1016 cross

Root type	Trait	Chromosome no.	QTL peak position (cM)	Nearest marker position (cM)	LOD		R <sup>2</sup> value <sup>c</sup> (%)		Mean additivity <sup>d</sup>
					Joint <sup>a</sup>	Q×E <sup>b</sup>	Experiment 1	Experiment 2	
Primary	Weight (mg)	1	38	UMC11A (39)	4.9	1.4	4.0	1.1	-1.16
		5	113	PGAMCGT165 (112)	6.3	0.9	6.2	0.7	-1.70
		9	13	CSU134C (13)	3.9	2.9	0.4	3.5	0.70
Seminal	Weight (mg)	3	41	PGAMCCA250 (41)	5.3	0.1	2.8	2.1	3.73
		7	20	UMC5B (20)	4.5	0.5	2.7	2.6	1.93
		10	76	UMC49B (80)	4.8	3.5	1.8	2.3	-0.55
PrAx	Length (cm)	1	13	PGAMCCC210 (13)	4.0	0.1	11.8	3.2	-1.31
		4	119	PGAMCTT430 (119)	3.6	2.1	2.5	1.2	1.10
		7	20	UMC5B (20)	4.9	1.3	3.6	1.2	1.44
		10	63	PGAMCTT250 (63)	5.3	2.8	5.1	9.6	-1.07
PrLat	Diameter (mm)	1	44	CSU145C (41)	6.1	0.7	14.2	16.6	-0.064
		1	162	CDO122A (158)	5.0	2.5	2.3	17.4	-0.056
		2	57	PGAMCCA590 (60)	4.2	0.4	1.7	1.4	0.073
		3	100	CSU58A (99)	4.2	0.6	6.7	3.3	0.041
		5	104	PGCMCTA260 (104)	3.7	0.6	8.3	8.4	-0.049
		10	63	PGAMCTT250 (63)	5.3	2.8	5.1	9.6	-1.07
PrLat	Length (cm)	1	93	PGAMCTA205 (92)	3.9	1.6	7.1	2.0	4.65
		3	96	UMC48C (95)	3.9	0.9	2.0	6.1	0.96
		5	109	PGAMCCA500 (109)	6.1	4.7	11.7	1.1	-5.46
		6	55	CSU116A (54)	4.3	2.0	5.7	4.1	-6.77
		9	11	PGAMCCA100 (10)	4.1	2.9	1.4	6.3	5.39
SeAx	Count (cts)	1	49	ASG45 (49)	5.6	1.8	9.2	8.8	0.39
		4	82	PGAMCTT300 (81)	3.6	3.2	0.8	6.3	-0.06
		5	123	UMC108 (129)	4.8	4.2	3.2	4.4	-0.09
		5	141	PGAMCGG330 (132)	4.9	4.8	0.6	6.2	-0.04
		10	84	UMC49B (80)	5.2	2.7	1.6	11.6	0.19
SeAx	Length (cm)	1	47	ASG45 (49)	4.3	0.1	9.3	9.7	3.63
		2	17	PGAMCTA210 (11)	4.1	1.9	8.4	2.1	3.44
		3	56	UMC16A (59)	3.9	3.2	0.6	0.8	3.50
SeAx	Diameter (mm)	1	81	UMC67 (83)	5.5	0.6	12.4	19.6	-0.064
		10	63	PGAMCTT250 (63)	5.3	2.8	5.1	9.6	-1.07

**Table 2** (continued)

Root type	Trait	Chromosome no.	QTL peak position (cM)	Nearest marker position (cM)	LOD		$R^2$ value <sup>c</sup> (%)		Mean additivity <sup>d</sup>	
					Joint <sup>a</sup>	Q×E <sup>b</sup>	Experiment 1	Experiment 2		
SeLat	Length (cm)	1	83	UMC67 (83)	4.4	0.8	10.2	6.8	2.71	
		1	159	CDO122A (158)	4.4	1.8	0.6	14.7	2.49	
		4	69	PGAMCTT300 (81)	4.3	2.5	2.2	0.9	-3.18	
		6	20	PGCMCGA450 (20)	7.9	7.8	2.1	2.1	0.33	
	Diameter (mm)	1	70	PGAMCCC280 (70)	5.4	0.7	6.4	13.4	20.8	2.35
		4	10	UMC156A (14)	11.1	10.5	1.7	8.8	0.008	
		7	74	PGAMCGG150 (72)	3.9	1.0	0.4	1.1	0.018	
							8.6	11.1	0.005	

<sup>a</sup>Joint analysis of the two experiments<sup>b</sup>QTL-by-experiment interaction<sup>c</sup> $R^2$  value, Phenotypic variance explained by each QTL<sup>d</sup>Additive effects represent the substitution of a Lo964 allele with a Lo1016 allele<sup>e</sup> $R^2$  of the final multiple regression model<sup>f</sup>Sum of the additive effects

## Shoot and seed traits

### Phenotypic analyses

Mean values and the statistical analysis of shoot and seed traits are shown in Table 3. The parents significantly differed for the following traits: germination, leaf area/root length ratio, shoot/root DW ratio, shoot DW, leaf area, SPAD values of the second leaf, and  $\Phi_{PSII}$  of the second leaf. The differences among families were significant for all traits with the exception of Fv/Fm of the second leaf (Table 3). A transgressive segregation was noted for all shoot traits. Most of the  $h^2$  values were above 60% with the highest values (69%) for shoot DW and SPAD values of the second leaf. The lowest  $h^2$  values were found for the germination index (43%) and for Fv/Fm of the second leaf (21%). There was a significant correlation between the SPAD values and  $\Phi_{PSII}$  of the F<sub>2:4</sub> families ( $r=0.54^{***}$  for leaf one and  $r=0.68^{***}$  for leaf two). With respect to the influence of the carbohydrate sources on biomass accumulation, there was a significant correlation between  $\Phi_{PSII}$  and plant DW ( $r=0.46^{***}$ ) but not between seed DW and plant DW.

### QTL analyses

The 20 QTL identified for seed and shoot traits are shown in Table 4. One QTL was detected for germination percentage, three for the germination index, two for the leaf area, three for the plant DW, four for  $\Phi_{PSII}$ , and seven for SPAD values. The QTL could be grouped into 15 chromosomal regions, of which three influenced at least two of the investigated traits. The highest joint LOD scores above six were observed for the leaf area,  $\Phi_{PSII}$ , and the plant DW on chromosome 8(86), 10(61), and 10(83), respectively. Depending on the experiment and the trait,

10.0 to 37.1% of the phenotypic variability among the families was explained by a final multiple regression model. The SPAD values were positively associated with  $\Phi_{PSII}$  (chromosome 10(56–61) and 10(81–82)). Among the three QTL for plant DW, two were positively associated with the SPAD values and  $\Phi_{PSII}$  (chromosome 10(56–61), 10(81–83)) and one with the leaf area (chromosome 8(84–86)). The positive alleles for these QTL were contributed by Lo964 in all cases.

### Root traits and their relation to shoot and seed traits

The parental lines differed significantly for the leaf area/root length ratio since Lo 964 had a significant higher overall root length than Lo1016 (98 cm vs. 82 cm) (Table 1) and a significant lower leaf area (17 cm<sup>2</sup> vs. 24 cm<sup>2</sup>) (Table 3). The leaf area/root length ratio of the F<sub>2:4</sub> families was most closely related to the PrLat and SeLat length ( $r=0.72^{***}$  and  $0.50^{***}$ , respectively), while the shoot/root DW was related most closely to the PrAx and SeAx diameter ( $r=-0.32^{***}$  and  $-0.29^{***}$ , respectively) (data not shown).

A hierarchical cluster analysis based on the Ward's minimum variance method was performed for the root, shoot and seed traits (Fig. 3). Those traits that were obtained as the sum or ratio of others and those that were very strongly correlated were excluded from the analysis. Fv/Fm of the second leaf was also excluded because the variation for this trait was not significant among the families, and grain weight was excluded as an outlier in the first step of the cluster analysis based on the single-linkage method. In general, there were two main clusters: one grouping all the root diameters, the other grouping most of the root lengths and counts. The SPAD values and  $\Phi_{PSII}$  formed clusters with the diameters, while plant DW and leaf area formed clusters with the root length. Both the

**Table 3** Summary statistics for seed, shoot and photosynthesis-related traits of the parental inbred lines and their F<sub>2:4</sub> segregating offspring. For more detailed information on the parameters, see Table 1

Trait	Parental means			F <sub>2:4</sub> families				CV <sub>G</sub> (%) <sup>a</sup>	h <sup>2</sup> (%) <sup>b</sup>
	Lo 964	Lo 1016		Maximum	Minimum	Mean			
Seed									
Grain weight (mg) <sup>c</sup>	237	271	NS <sup>d</sup>	343	189	277	**** <sup>e</sup>	9	–
Germination Index (d)									
Germination (%)	2.52	3.62	NS	5.61	2.81	3.62	***	7	43
Plant									
Overall dry weight (mg)	81.9	51.9	**	1000	17.8	64.6	***	20	63
Leaf area/ root length									
Shoot/ root dry weight	102.1	105.3	NS	149.4	36.3	105.3	***	14	66
Shoot	0.19	0.30	***	0.46	0.15	0.26	***	18	68
Shoot dry weight (mg)	1.61	2.57	***	3.27	1.26	2.29	***	11	63
Leaf area (cm <sup>2</sup> )									
Photosynthesis	63.0	75.6	**	111.2	21.3	72.8	***	15	69
SPAD values									
Leaf 1 $\Phi_{PSII}$	17.2	24.1	***	31.4	13.0	22.2	***	12	63
Leaf 2 $\Phi_{PSII}$	27.0	20.7	NS	30.8	12.7	23.6	***	11	58
SPAD values									
Leaf 1 $\Phi_{PSII}$	0.25	0.28	NS	0.40	0.10	0.25	***	21	66
Leaf 2 $\Phi_{PSII}$	22.5	11.7	***	27.0	6.28	18.3	***	19	69
SPAD values									
Leaf 2 $\Phi_{PSII}$	0.28	0.19	***	0.37	0.08	0.23	***	16	56
Fm									
Leaf 2 Fv/ Fm	0.70	0.62	NS	0.75	0.36	0.63	NS	5	21

\* , \*\* , \*\*\*Comparisons between mean values are significant at  $P \leq 0.05$ , 0.01 and 0.001, respectively. NS, Not significant

<sup>a</sup> The genetic coefficient of variation was calculated from the REML estimate of variance

<sup>b</sup>  $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2 / 2 + \sigma^2 / 4)$ , where  $\sigma_G^2$ ,  $\sigma_{GE}^2$ , and  $\sigma^2$  are the REML estimates of variance for the genotype, genotype  $\times$  experiment, and error, respectively

<sup>c</sup> Average seed weight  $\pm 10\%$

<sup>d</sup> Comparison of parental inbreds

<sup>e</sup> Comparison of F<sub>2:4</sub> families

SPAD values and  $\Phi_{PSII}$  were frequently related to the root diameter traits ( $r=0.17^*$  to  $0.30^{**}$ ) but not to root length. In contrast, plant DW and leaf area were always significantly correlated to root length ( $r=0.23^{**}$  to  $0.42^{**}$ ). It is noteworthy that the germination index clustered with the PrLat length. There were significant correlations between PrLat length and germination

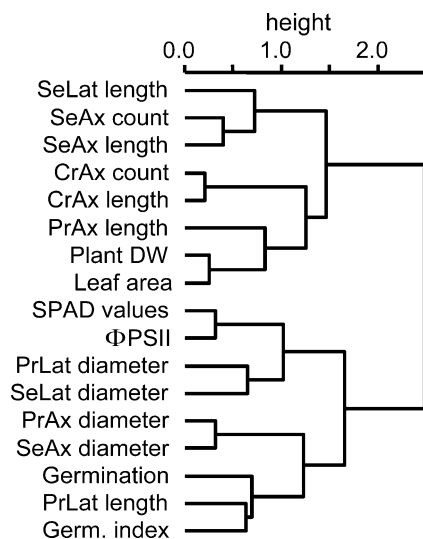
percentage ( $r=0.33^{**}$ ) and germination index ( $r=0.38^{**}$ ). The germination percentage was also positively correlated with the PrAx and SeAx diameter ( $r=0.39^{**}$  and  $0.24^{**}$ , respectively).



**Table 4** Putative QTL for seed (LOD>2.8) and shoot traits (LOD>3.6) of 168 F<sub>2:4</sub> families of the Lo964 × Lo1016 cross

Trait	Chromosome No.	QTL peak position (cM)	Nearest marker position (cM)	LOD		R <sup>2</sup> value % <sup>c</sup>		Mean additivity <sup>d</sup>					
				Joint <sup>a</sup>	Q×E <sup>b</sup>	Experiment 1	Experiment 2						
Germination (%)	4	110	CDO127A (109)	4.6	–	10.8	–	–10.64					
						10.8 <sup>e</sup>	–	–10.64 <sup>f</sup>					
Germination index (d)	5	109	PGAMCCA500 (109)	5.2	–	14.2	–	0.24					
	6	2	UMC59A (2)	2.9	–	2.9	–	–0.16					
	6	57	PGAMCTA175 (58)	4.1	–	7.9	–	0.26					
Leaf area (cm <sup>2</sup> )	1	159	CDO122A (158)	3.6	0.9	4.1	8.8	1.31					
						23.6	–	–					
						14.5	10.0	–0.03					
Plant DW (mg)	8	84	BNLG240 (71)	4.7	1.5	7.8	2.1	–5.87					
	10	61	PGAMCTT250	3.7	1.7	8.8	5.0	–6.05					
	10	83	UMC49B (80)	6.2	4.3	12.1	6.2	–4.82					
Φ <sub>PSII</sub>	1	8	PGAMCCC210 (13)	4.3	2.8	2.5	7.6	0.014					
						17.4	20.4	–0.024					
						10	10	PHP20075A (9)	4.3	3.4	0.1	8.2	0.010
						10	61	PGAMCTT250	6.1	2.9	11.7	3.2	–0.026
SPAD values	10	82	UMC49B (80)	6.0	2.5	10.7	6.8	–0.022					
						37.1	36.9	–3.49					
						1	71	PGAMCCC280 (70)	4.0	1.9	6.6	6.0	–1.40
						1	148	CDO122A (158)	3.9	0.3	7.9	5.6	–2.13
						3	42	PGAMCCA250 (41)	4.3	1.1	4.4	4.2	–0.78
						4	54	PGAMCTA345 (45)	4.9	3.1	1.7	4.4	2.67
SPAD values	5	122	UMC108 (129)	4.1	0.8	4.2	7.0	1.90					
						10	56	UMC130 (55)	5.0	1.0	12.5	0.7	–1.93
						10	81	UMC49B (80)	5.5	2.6	7.0	6.1	–1.82
						–	–	–	–	–	–	–	–
						–	–	–	–	–	–	–	–

<sup>a–f</sup>For more detailed information on the parameters, see Table 2



**Fig. 3** Dendrogram obtained from hierarchical cluster analysis of seed, root and shoot traits of 168 F<sub>2:4</sub> families of the Lo964 × Lo1016 cross. The cluster analysis was performed using the correlation distance matrix and Ward's minimum variance method

## Discussion

### Root traits

The population investigated in this study had already been shown to have genetic variability for root morphological traits (Landi et al. 2001), and QTL had been successfully mapped for these traits under optimum temperature (Tuberosa et al. 2002b). In the present study, the root structural parameters were investigated to establish differences among genotypes in cold conditions. The lateral root length is the most reliable trait for establishing differences among the genotypes due to its high CV<sub>G</sub> and considerably high *h*<sup>2</sup>. The lowest CV<sub>G</sub> was observed for root diameters and root counts. In contrast to the observations of Cahn et al. (1989), we found that the relationship between the diameter and the length of the roots was usually negative, possibly due to the effect of the low soil temperature, at which maize roots are known to grow thicker and less branched (Brouwer 1967; Cutforth et al. 1986; Stamp 1979). The negative phenotypic relationship between root length and root diameter was verified by four out of six loci. For example, an increase in PrAx diameter

was associated with a decrease in SeAx length on chromosome 1(44–47), and an increase in SeAx diameter was associated with a decrease in SeLat length on chromosome 1(81–83).

Hierarchical cluster analyses grouped the different root traits into distinct sub-clusters, and this finding is in accordance with their different genetic control. In fact, genes controlling the different root traits (e.g. *rtcs* and *rt1*) have been identified as have genes controlling the overall formation of lateral roots (e.g. *slr1*, *slr2*) (Feix et al. 2000). However, most of these genes are not mapped precisely enough to test their association with the QTL controlling the root traits in this study. The relatively high number of detected QTL regions and their distribution over the whole genome confirms the complexity of the inheritance of root traits, as reported by Tuberosa et al. (2002b). However, the different root types (primary and seminal roots) were not always completely independently inherited. For example, an increase in SeAx length and count was associated with a decrease in PrAx diameter on chromosome 1(44–49), and an increase in SeLat length was associated with a decrease in PrAx and PrLat diameters on chromosome 1 (159–164). This could be due to close linkage but could also have pleiotropic causes—for example, a result of the compensation for the poor development of one root type by another.

The small differences between the  $R^2$  values of the final simultaneous fit and the sum of the  $R^2$  values of the individual QTL indicate that epistasis played only a minor role in controlling the traits. The fairly balanced number of positive alleles contributed by each parent supports the observation of transgressivity, as indicated by the larger range of variation found for the  $F_{2,4}$  families compared to the difference between the parental lines. However, this was not the case for traits that the parents differed strongly for: positive alleles for SeAx and SeLat length were almost exclusively contributed by Lo1016, while those for PrLat diameter were almost exclusively contributed by Lo964.

#### Stability of root traits across environments

Differences in the organisation of the embryonic root system of Lo964 and Lo1016 were reported by Landi et al. (2001) and Sanguineti et al. (1998); the QTL controlling root traits at close-to-optimum temperature conditions were mapped by Tuberosa et al. (2002b). A comparison of our results with those previously obtained with the same genetic material allows us to discuss the effect of environmental conditions on QTL detection. However, we may not have found all QTL controlling the traits, given the limited size of the population examined (Beavis 1998; Openshaw and Frascaroli 1997) and the associated problems due to genotypic sampling (Utz et al. 2000). A comparison of the root morphology of the two parents grown at close-to-optimum temperature in hydroponics (Landi et al. 2001; Sanguineti et al. 1998) with that of the parents grown in the sand/vermiculite substrate at low temperature shows that the organisation of the root system

is stable across environments. The parents expressed a nearly identical phenotype in both environments, but overall plant growth was influenced by the different growth conditions realised in the two studies. In fact, plants at the two-leaf stage in hydroponics (Landi et al. 2001) had about the same shoot and root DW as plants at the one-leaf stage grown in the sand/vermiculite substrate, and the diameters of the PrAx roots were about 0.4–0.5 mm thicker in the sand/vermiculite substrate than in hydroponics. Even though thicker roots are more regularly found at low temperatures than at the optimal temperature (Brouwer 1967; Cutforth et al. 1986; Stamp 1979), these differences in root diameter may also be due to the different substrates. The similarity of root traits grown in hydroponics and in sand/vermiculite suggests the possibility of finding common QTL controlling the traits under both conditions. However, Jansen et al. (1995) pointed out that the chances of detecting a QTL in several environments is small, even when the QTL-by-environment interaction is not significantly relevant. To compare the QTL found in this study with those detected by Tuberosa et al. (2002b), we only considered directly comparable traits (diameter and length of PrAx and weight of primary and seminal roots): seven of the QTL reported by Tuberosa et al. (2002b) were confirmed to have the same genomic location, all of them with the same sign of additive effects. Of these, two of the three regions with the highest LOD values found by Tuberosa et al. (2002b) were confirmed in this study—the locus for seminal root weight and the length and count of SeAx on chromosome 1(44–49) (LOD=3.3 for seminal weight) and the locus for primary root weight on chromosome 1(34–38). The third major QTL detected by Tuberosa et al. (2002b) for PrAx diameter on chromosome 1(89) fell just below the LOD threshold in the present study. QTL for root traits have been identified in various other populations at the same positions that we found to control the primary root weight at 38 cM (bin 1.03) and the PrAx diameter at 89 cM (bin 1.06) (Tuberosa et al. 2002a). Apart from the above mentioned loci on chromosome 1, five others were detected in both studies: one on chromosome 2(56–57) for PrAx diameter, two on chromosome 7(12–22) for PrAx length and the seminal root weight, one on chromosome 9 (10–13) for primary root weight, and one on chromosome 10(74–76) for seminal root weight. As most of the matching QTL in this study were characterised by LOD scores of above four and weak QTL-by-experiment interactions, they can be considered to be very stable.

#### Shoot and seed traits

Seed vigour, germination and germination index were evaluated at close-to-optimum conditions to reduce the effect of cold stress on plant performance. Nevertheless, there was considerable variation in these traits, which provided us with the possibility to investigate their genetic control. Two of QTL found for these traits confirmed those found by E. Frascaroli et al. (unpublished data) in a study

investigating cold tolerance at germination in the same mapping population. One QTL for percentage germination on chromosome 4(110) matched a QTL for germination at 9°C and 25°C, and one QTL for germination index on chromosome 5(102–109) matched one for germination at 9°C and the germination index in the unpublished study of E. Frascaroli et al.

In agreement with the results of Landi et al. (2001), seed weight was not associated with early plant growth in this study (data not presented). In contrast,  $\Phi_{\text{PSII}}$ , which is well correlated with the quantum yield of carbon fixation under controlled conditions (Fracheboud et al. 2002), was correlated with plant DW. These results are in agreement with an earlier study on 21 inbred lines where plant DW was also more closely correlated with  $\Phi_{\text{PSII}}$  than with seed DW (Hund 2003). This finding suggests that a strong photosynthetic performance is an important attribute for vigorous early growth at low temperatures as early as at the one-leaf stage. The relationship between  $\Phi_{\text{PSII}}$  and plant DW was confirmed at two loci on chromosome 10 that explained together between 10% and 20% of the phenotypic variance, depending on the experiment.

#### Root traits and their relation to shoot and seed traits

Root/shoot relationships are often used to elucidate differences in biomass or structure of the different organs. Our study shows that differences in the leaf area/root length ratio of genotypes are mainly due to changes in the structure of the lateral roots, while differences in the shoot/root DW ratio are due to changes in the thickness of the axile roots. In the cluster analysis, because traits showing negative relationships were considered to be very distant, the root diameters formed a cluster far from the cluster of root lengths as these traits were usually negatively correlated. The photosynthesis-related traits like SPAD values and  $\Phi_{\text{PSII}}$  were clustered with root diameter and, thus, are related to thicker roots. This relationship was confirmed for SeLat diameter and SPAD values on chromosome 1(70–71), there was a close association between the PrAx and PrLat diameter and SPAD values on chromosome 1(148–164). A close relationship between root traits and photosynthesis-related traits and plant DW was not observed in this study. This finding indicates that the root morphological characteristics (intensive versus extensive rooting) alone can not be utilised to improve vigour in terms of an increased photosynthetic ability and plant DW at low temperatures. However, there was a clear influence of seed vigour on PrLat length that could be confirmed on the genetic level on chromosome 5(109) and chromosome 6(55–57) by a co-location of PrLat length and the germination index. In particular, the locus on chromosome 5(109) controlled 12% of the phenotypic variability for the germination index and 14% of the variability of for PrLat length in experiment 1. E. Frascaroli et al. (unpublished data) found a QTL for cold tolerance at germination and the germination index at 9°C at the same locus. The fact that the QTL was detected in

this study for germination at the optimum temperature could be due to the low temperature during imbibition. Since this locus not only influences germination but also root structure, it is likely that it is a key locus for both seed vigour and early root morphology.

#### Conclusion

The population we examined in this study segregates for a large number of independent QTL controlling the vigour of both the root and the shoot traits at low temperature. The overall organisation of the root system and the major QTL detected proved to be stable across environments, as shown by a comparison of our results with those found in earlier studies in hydroponics.  $\Phi_{\text{PSII}}$  and SPAD were identified as promising traits for the indirect selection of genotypes with improved plant DW at low temperatures and were also associated with an increase in the root diameters but not with root length. The length of the primary lateral roots was positively associated with germination speed, indicating a genetic relationship between seed vigour and the morphology of the embryonic root system.

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