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Publication date:
2011-07

Permanent link:
https://doi.org/10.3929/ethz-b-000037494

Originally published in:
Theoretical and Applied Genetics 123(2), https://doi.org/10.1007/s00122-011-1586-8

Theor Appl Genet (2011) 123:327–338
DOI 10.1007/s00122-011-1586-8

ORIGINAL PAPER

QTL involved in the partial restoration of male fertility of C-type cytoplasmic male sterility in maize

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Received: 1 December 2010 / Accepted: 24 March 2011 / Published online: 9 April 2011

Abstract Partial restoration of male fertility limits the use of C-type cytoplasmic male sterility (C-CMS) for the production of hybrid seeds in maize. Nevertheless, the genetic basis of the trait is still unknown. Therefore, the aim to this study was to identify genomic regions that govern partial restoration by means of a QTL analysis carried out in an F_2 population ($n = 180$). This population was derived from the Corn Belt inbred lines B37C and K55. F_2BC_1 progenies were phenotyped at three locations in Switzerland. Male fertility was rated according to the quality and number of anthers as well as the anthesis-silking interval. A weak effect of environment on the expression of partial restoration was reflected by high heritabilities of all fertility-related traits. Partial restoration was inherited like an oligogenic trait. Three major QTL regions were found consistently across environments in the chromosomal bins 2.09, 3.06 and 7.03. Therefore, a marker-assisted counter-selection of partial restoration is promising. Minor QTL regions were found on chromosomes 3, 4, 5, 6 and 8. A combination of partial restorer alleles at different QTL can lead to full restoration of fertility. The maternal parent was clearly involved in the partial restoration, because the restorer alleles at QTL in bins 2.09, 6.04 and 7.03 originated from B37. The three major QTL regions collocated with other restorer genes of maize, a phenomenon, which seems to be typical for restorer genes. Therefore, a study of the clusters of restorer genes in maize could lead to a better understanding of their evolution and function. In this respect, the long arm of chromosome 2 is particularly interesting, because it harbors restorer genes for the three major CMS systems (C, T and S) of maize.

Communicated by R. Johnson.

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Introduction

C-type cytoplasmic male sterility (CMS) is today the most widely applied form of CMS for the production of hybrid seeds in maize. However, an important shortcoming of C-CMS is the frequent occurrence of partially restored plants, which bear fewer anthers and shed less pollen than fully fertile plants. Anthers are often misshaped and usually emerge after silking (Tracy et al. 1991). Partially restored inbred lines cannot be used as seed parents, because even a low level of pollen shedding can lead to self-pollination of the maternal parent and, consequently, to impure hybrid seeds. Furthermore, if CMS were employed to contain transgenic pollen, it would be necessary to prevent partial restoration (Munsch et al. 2009).

The environment apparently has a strong impact on the expression of partial restoration (Duvick 1956; Tracy et al. 1991; Weider et al. 2009). A C-CMS inbred line may be sterile at one location or in a particular year but may shed considerable amounts of pollen under different environmental conditions. In general, climatic factors like temperature and photoperiod are assumed to be of major importance (Kaul 1988). Humid and cool environments are assumed to be conducive to the expression of male fertility, whereas in dry and hot locations, sterility is maintained (Duvick 1965; Tracy et al. 1991). Even though partial restoration has important practical implications, its genetic

basis is still unknown. In previous studies, the strong environmental effect made it impossible to draw conclusions about the number of involved genes (Tracy et al. 1991). However, partial restoration is clearly a heritable trait, because selection of progenies with a reduced partial restoration is usually successful over few generations (Gontarovskii 1974; Tracy et al. 1991). Partial restoration might be governed by multiple factors, which may have an effect in the absence of genes responsible for full restoration (Tracy et al. 1991; Has 2002). Complementary interactions between paternal and maternal factors probably play an important role (Gontarovskii 1974; Vidakovic 1988; Sotchenko et al. 2007).

To select against partial restorer alleles in the breeding germplasm, it is important to gain a better understanding of the mode of inheritance. If major genomic loci for partial restoration were revealed, marker-assisted selection would enhance the development of maternal inbred lines, because the trait might not be expressed every year or at each location, depending on the environmental conditions. The aim of this study was, therefore, to conduct a QTL analysis to identify major genomic regions involved in the partial restoration of male fertility.

Materials and methods

Plant material

Mapping of QTL was carried out in an F_2 mapping population, derived from a cross between the C-CMS inbred line B37C and the restorer inbred line K55. Seeds of the parental lines were obtained from the Maize Genetics Cooperation Stock Center at the University of Illinois (Urbana Champaign, IL, USA). The mapping population segregated for the major restorer gene *Rf4* and an unknown number of weak restorer genes, which partially restore male fertility. *Rf4* is located on the 5′ telomeric end of chromosome 8 (Sisco 1991; unpublished results). The *Rf4* allele is dominant over the *rf4* allele (Kheyr-Pour 1981; unpublished results). It has a strong effect on the restoration of fertility and can mask the effect of the partial restorer genes. Therefore, 198 F_2 plants, carrying the non-restoring *rf4* allele, were selected by means of a closely linked marker (proprietary to KWS Saat AG, Einbeck, Germany) to map the unknown partial restorer QTL in a background of partial restorer genes (Fig. 1).

Phenotypic values for QTL mapping were obtained from F_2BC_1 progenies, which were generated by backcrossing the selected F_2 plants to the male-fertile version of the maternal line B37 carrying a normal cytoplasm (B37N). This approach was comparable to the $F_{2:3}$ design described by Soller et al. (1990), the difference being that backcross progenies rather than selfing progenies were generated, because partially fertile and sterile plants produce little or no pollen (Fig. 1).

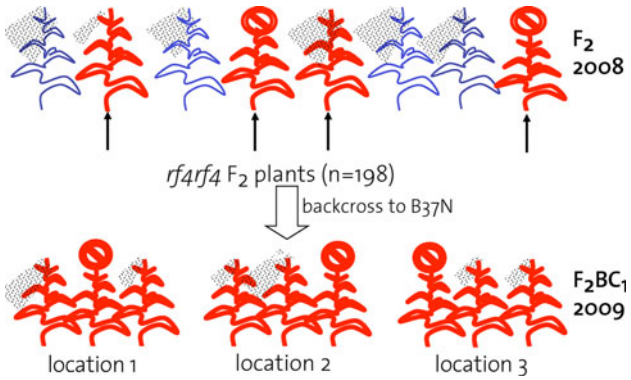

Fig. 1 Construction of the QTL mapping population: 198 *rf4rf4* plants (*red*, *bold*) were selected from a complete F_2 population, 180 were genotyped. F_2BC_1 progenies were generated by backcrossing the selected F_2 plants to the male-fertile version of the maternal parent, B37N. F_2BC_1 progenies were phenotyped at three locations in Switzerland (color figure online)

Field experiments

The F_2 population was grown at the experimental station of the Swiss Federal Institute of Technology Zurich at Eschikon in 2008. Twenty-five kg/ha P and 165 kg/ha K were applied before sowing, and 120 kg/ha N was applied in two portions of 60 kg/ha 3 and 6 weeks after sowing. Kernels were sown at intervals of 20 cm within a row and 75 cm between the rows. The plants were germinated under a fleece in order to accelerate early development. Leaf samples were taken at the five-leaf stage from each plant to extract DNA. In 2009, the F_2BC_1 progenies of 198 selected F_2 plants were grown in Cadenazzo (CAD), Delley (DEL) and Eschikon (ESH). Table 1 gives the descriptors for these environments. The environments were chosen because of their different climatic conditions. CAD, located on the Magadino plain of Ticino (southern Switzerland), is characterized by a higher mean temperature, higher humidity and more rainfall than DEL, which is located in the western part of the country. ESH is located in northern Switzerland and has a lower mean temperature than CAD and more rainfall than DEL.

All the experiments were laid out as alpha (0.1) lattices with two replications, 20 blocks and 10 plots per block. The F_2BC_1 families were tested together with two entries for the parental lines B37C and K55. At ESH and CAD, 20 plants were sown in one plot, whereas at DEL, 16 plants were sown per plot. Seeds at DEL and ESH were sown with a plot seeder, whereas at CAD, the seeds were sown manually with jab planters. At DEL, plants were germinated under a fleece. At CAD, plants were irrigated with

Table 1 Description of the experimental locations Cadenazzo (CAD), Delley (DEL) and Eschikon (ESH)

	CAD	DEL	ESH
Coordinates	46°8′N, 8°55′E	46°55′N, 6°57′E	47°26′N, 8°40′E
Elevation (m a.s.l.)	203	500	546
Soil[a]	Sandy colluvisol	Chromic luvisol	Eutric cambisol
Sowing date	08.05.2009	22.04.2009	01.05.2009
Mean precipitation[b] (mm)	887	796	828
Mean temperature[b] (°C)	19	16	16

[a] Soil classification according to IUSS Working Group WRB (2007)

[b] Long-term mean during the vegetation period from April to September

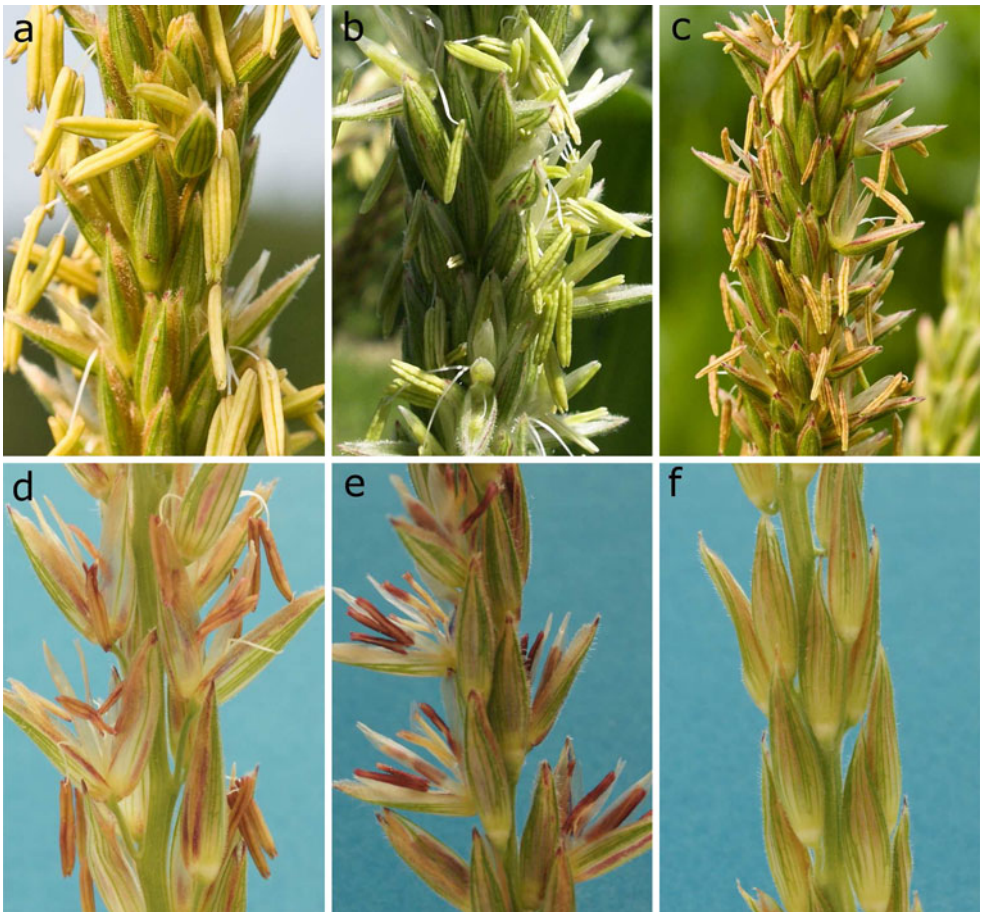

Fig. 2 Scale of anther quality: **a** 1, normal dehiscent anthers and full shed of pollen; **b** 2, some stunted anthers and nearly full shed of pollen; **c** 3, many stunted anthers and reduced shed of pollen; **d** 4, only stunted anthers, nearly no shed of pollen; **e** 5, completely empty anthers and no shed of pollen; **f** no emergence of anthers

30 mm of water at the six-leaf stage. Fertilization and plant protection were carried out according to the local practice.

Phenotyping male fertility

Male fertility of each F_2 and F_2BC_1 plant was rated according to the quality and number of anthers as well as the anthesis-silking interval (ASI). Plant height was considered to be a general parameter of plant vitality. ASI was defined as the difference between the date of silking and anthesis. The quality of the anthers (anther quality) was rated on a scale of 1 to 6 (Fig. 2): 1, normal, dehiscent anthers, full shed of pollen; 2, some stunted anthers, full shed of pollen; 3, many stunted anthers and reduced shed of pollen; 4, only stunted anthers, nearly no shed of pollen; 5, empty anthers and no shed of pollen; 6, no emergence of

anthers. The number of anthers (anther quantity) was rated on a scale of 1 to 6, too: 1, more than 75% anther emergence; 2, more than 50–75% anther emergence; 3, more than 25–50% anther emergence; 4, more than 5–25% anther emergence; 5, more than 0–5% anther emergence; 6, no emergence of anthers (equivalent to a score of 6 for anther quality). Plants were considered to be partially fertile when anther quality and quantity had scores equal or higher than 3. Sterile plants did not bear anthers during the first 3 weeks after silking. In all experiments, only the ten plants located in the center of a plot were phenotyped to minimize border effects on time of flowering. Observations were made in the morning when the anthers were fresh and filled with pollen. The tassel of each plant was examined every other day, and all the plants were phenotyped three to four times during male flowering. The final scores for anther quality and quantity of a plant corresponded to the average quality scores and the last quantity score, respectively.

Statistical analysis of field experiments

The experimental designs were evaluated by means of the R package ASReml (Butler et al. 2007; R Development Core Team 2009). Missing values were replaced by predicted values, which were estimated as a consequence of fitting the model. Only few single-plant measurements were missing (3.3, 4.2, 3.2 and 2.2% of the measurements of anther quality, anther quantity, ASI and plant height, respectively). Analyses of variance and the best linear unbiased predictors (BLUPs) of each F_2BC_1 family were calculated from the plot raw data for anther quality, anther quantity, ASI and plant height in each individual trial as well as across environments. The mixed effects model used to analyze each individual trial was:

$$y_{ijk} = \mu + f_i + r_j + b(r)_{jk} + (fr)_{ij} + e_{ijk} \quad (1)$$

where y_{ijk} is the predictor of the ith family (f) in the jth replication (r) and the kth block (b) within the jth replication, μ the intercept and e_{ijk} the residual error variance. Replications were assumed to be fixed effects and incomplete blocks and families were assumed to be random. Within-location heritabilities (sometimes referred to as "repeatabilities") were calculated according to:

$$h^2_{\mathrm{w}} = \frac{\sigma^2_{\mathrm{f}}}{\sigma^2_{\mathrm{f}} + \frac{\sigma^2_{\mathrm{fr}}}{R} + \frac{\sigma^2_{\mathrm{e}}}{N}} \quad (2)$$

where h^2_{w} is the within-location heritability, σ^2_{f} the variance of the families, σ^2_{fr} the interaction variance of families × replications and σ^2_{e} the residual error variance. R and N denote the number of replications and the nominal number of plants per plot, respectively. The model for the analysis across environments was:

$$y_{ijkl} = \mu + f_i + l_j + (fl)_{ij} + r(l)_{jk} + b(lr)_{jkl} + (flr)_{ijk} + e_{ijkl} \quad (3)$$

where y_{ijkl} is the predictor of the ith family in the jth environment (l), in the kth replication and in the lth block within the kth replication, μ the intercept and e_{ijkl} the residual error variance. Environments were assumed to be fixed, all the other factors were assumed to be random. The heritability was calculated according to:

$$h^2 = \frac{\sigma^2_{\mathrm{f}}}{\sigma^2_{\mathrm{f}} + \frac{\sigma^2_{\mathrm{fl}}}{L} + \frac{\sigma^2_{\mathrm{flr}}}{LR} + \frac{\sigma^2_{\mathrm{e}}}{N}} \quad (4)$$

where h^2 is the heritability, σ^2_{f} the variance of the families, σ^2_{fl} the interaction variance of families × environments, σ^2_{flr} the interaction variance of families × environments × replications and σ^2_{e} the residual error variance. L, R and N denote the number of environments, replications and measured plants per plot, respectively.

The phenotypic coefficients of correlation (Pearson's) and significance levels were determined by linear regressions in R based on standardized BLUPs (mean 0, standard variation 1).

Genotyping

180 of 198 F_2 plants were randomly selected for genotyping. A whole-genome scan was carried out by Traitgenetics GmbH (Gatersleben, Germany) using a 1,536 SNP Illumina GoldenGate array (OPA GS0010903; Illumina Inc., San Diego, CA, USA) and whole-genome amplified DNA. The SNPs covered 512 loci that were evenly distributed over the maize genome (three SNPs per locus, belonging to different haplotypes). Furthermore, 672 SNP markers were analyzed on the Illumina Veracode BeadXpress platform multiplexing 384 SNPs in one reaction. The GoldenGate assays were performed according to the manufacturer's protocol and as described in Fan et al. (2003). The automatic allele calling for each locus was accomplished with the Illumina GenomeStudio Analysis Software. Both alleles of a SNP give different signals due to the allele-specific ligation to fluorescent products. Therefore, a genotype that is homozygous for one or the other SNP alleles gives one signal, whereas a heterozygous genotype gives both signals. All SNPs were checked for good separation of the homozygous and heterozygous signals and were manually re-scored when errors in calling the homozygous or heterozygous signals were found. 330 polymorphic high-quality SNP markers were chosen for linkage mapping.

Construction of the genetic linkage map

The linkage map was constructed with the software Joinmap 4.0 (van Ooijen 2006). Five of 180 individuals were excluded from this procedure because of an elevated mean number of crossing overs per individual. This was probably due to genotyping errors. χ^2 values were calculated for all individual markers to detect deviations in gametic segregation from the expected Mendelian 1:2:1 ratio for an F_2 population ($\alpha = 0.05$). Bonferroni correction was considered for multiple tests. A minimal LOD (log likelihood of the odds) of 3.0, a maximal recombination fraction of 0.5 and a maximal jump of 5 were used for clustering of markers. After construction of the map, the data files were screened for putative double recombinants based on the "genotype probabilities" calculated by Joinmap. Genotypes with a low probability were verified or corrected by reexamining the genotypic data. Once the most probable order of the markers was obtained, genetic distances were estimated for each linkage group applying the Kosambi mapping function (Kosambi 1944). The genetic positions of all SNP markers were projected onto the IBM neighbors 2008 reference map (Schaeffer et al. 2008).

QTL analysis

Composite interval mapping (CIM; Zeng 1994) was performed with the software PLABQTL (Utz and Melchinger 2000) for each individual trait in each environment (individual QTL analyses) as well as in a combined approach across environments (joint analyses). The minimal LOD required to declare a QTL significant was obtained empirically for each trait by 1,000 permutation tests (Churchill and Doerge 1996). The resulting LOD thresholds ($\alpha = 0.05$) varied from 4.45 to 4.71, depending on the trait, with an average of 4.70. The latter was applied as the significance threshold for the detection of QTL in all analyses. CIM was used with an "F-to-enter" value of 3.5 for the step-wise regression to preselect cofactors. Subsequently, the "cov SELECT" option was applied, which takes all the preselected markers as cofactors. However, if closely adjacent markers were selected as cofactors, then one of them was removed manually. The support interval of a QTL was defined as the length of the segment of the chromosome, over which the LOD at the peak decreased by half.

The effect of genotypic sampling on the estimation of QTL was tested by 200 rounds of fivefold cross-validation using the "cross-validate" option of PLABQTL. In each round, positions and effects of QTL of 80% of the genotypes were estimated, and a validation was performed with the remaining 20%.

The percentage of phenotypic variance explained by all QTL identified for a given trait was estimated by the adjusted coefficient of determination (adjusted R^2; Hospital et al. 1997). The percentage of phenotypic variance explained by an individual QTL was calculated by the normed partial correlation coefficient of determination (npartR^2). With such a normalization, the sum across all QTL is equal to the adjusted R^2 (Zhu et al. 2004).

The additive effect of a QTL was calculated as the median of the additive effects obtained from 1,000 cross-validation runs, taking into account only those QTL, whose peaks were located within the LOD support interval obtained by CIM. The additive effects as such were calculated as half the difference between the mean of the homozygous *RfRf* class and the mean of the homozygous *rfrf* class. However, since the genetic structure of F_2 parents and their F_2BC_1 progenies differed, the additive effects were underestimated by half. The explanation is that the F_2BC_1 progeny of an F_2 plant with the genotype AA at a given locus is supposed to be 100% heterozygous (Aa), and the progeny of an F_2 plant, heterozygous at a given locus (Aa), is supposed to segregate into 50% heterozygous (Aa) and 50% homozygous (aa) plants. Therefore, the additive effects obtained from PLABQTL were corrected by multiplying the values by two. Dominance effects could not be tested in the backcross design of this experiment. Epistatic interactions between additive effects were analyzed in ICIM (Li et al. 2007), which detects interactions between QTL with insignificant main effects.

Results

Climatic conditions

Temperature during the vegetation period in 2009 was close to the long-term mean in all environments (Table 2). CAD received the highest temperature. Rainfall differed widely among locations. CAD had more rainfall than the long-term mean and three times more than at DEL, the driest location. At DEL and ESH, there was less rainfall in spring and during flowering than the long-term mean. The patterns of rainfall at DEL and ESH were similar, and rainfall was more evenly distributed than at CAD where rainfall was seldom but very intense. At CAD, a wet early season, resulting in loss of plants due to water logging, was followed by a dry period in May and June, during which irrigation was required.

The maternal parent was partially restored

The maternal inbred line B37C was partially restored in both years of the study. The level of restoration differed from plant to plant but full restoration was never achieved. As a spot check, pollen of partially restored B37C plants

Table 2 Monthly mean temperature and precipitation at Cadenazzo (CAD), Delley (DEL) and Eschikon (ESH) and the long-term mean (ltm)

	Temperature (°C)						Precipitation (mm)					
	CAD		DEL		ESH		CAD		DEL		ESH	
	2009	ltm	2009	ltm	2009	ltm	2009	ltm	2009	ltm	2009	ltm
April	13	13	11	10	12	10	296	136	18	78	22	115
May	19	17	16	14	16	14	45	114	30	83	102	136
June	20	22	17	18	17	18	213	124	84	68	144	116
July	22	23	19	20	19	19	251	185	98	82	176	167
Aug	22	21	20	18	20	18	240	182	53	108	99	202
Sept	19	18	16	15	16	15	93	147	36	77	58	92

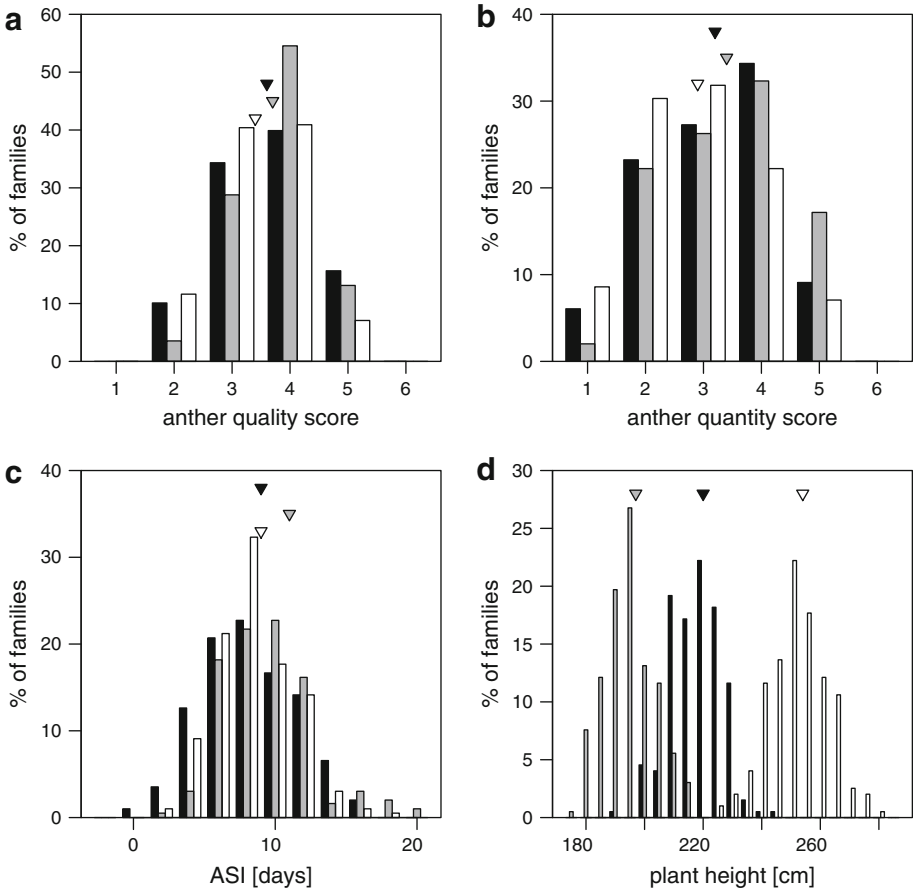

Fig. 3 Frequency distribution of F_2BC_1 family-based best linear unbiased predictors for **a** anther quality, **b** anther quantity, **c** ASI and **d** plant height at Cadenazzo (*black*), Delley (*gray*) and Eschikon (*white*). Means of individual trials: ▽

was examined under the microscope. Approximately half the pollen grains had aberrant shapes; the rest seemed to be viable (data not shown).

Segregation of male fertility and heritabilities

Male flowering started at temperature sums between 600 and 700°C and lasted for about 4 weeks in all environments. At CAD, anthesis started on July 19, in DEL on July 16 and in ESH on August 1. The majority of the 198 F_2BC_1 families segregated into partially restored and sterile plants. Approximately 15 families were nearly fully fertile, due either to recombination between *Rf4* and the marker used for selection or due to other genetic constellations. Although all these families reached full shed of pollen, anthers were slightly stunted (anther quality score 2) and male flowering started 3–7 days after silking, as it is typical for partially restored plants. None of the families was fully sterile.

The expression of restoration was similar among environments. The means of anther quality and anther quantity were not significantly different among the individual trials,

ranging from 3.4 to 3.7 for anther quality and from 2.6 to 3.1 for anther quantity (Fig. 3). Protogyny was strongly expressed in all environments, as it is a characteristic of partially restored plants. The average ASI was significantly longer at DEL (11 days) than at CAD and ESH (9 days), although the range of the values was similar. In contrast to the fertility-related traits, the mean plant height varied clearly among the environments, from 220 cm at DEL to 254 cm at ESH. The frequency distribution of all the traits followed approximate bell-shaped curves in all environments (Fig. 3). This suggests that multiple genes were involved in the partial restoration. h^2_w of the fertility-related traits was higher than 0.80, except that of anther quality at DEL (0.68). h^2_w of plant height was 0.77 at DEL and ESH but only 0.53 at CAD, probably resulting from an unequal development of the plants due to water logging after sowing.

The analysis of variance across families and environments gave highly significant differences among families for all traits. The variances of the location × family interaction and location × replication × family interaction were much smaller, however still significant (data not shown). Accordingly, h^2 of all the traits was high, but the h^2 of fertility-related traits (0.83–0.84) was higher than that of plant height (0.70).

Anther quality, anther quantity and ASI were closely correlated with each other in all environments. Whereas the phenotypic coefficients of correlation between anther quality and anther quantity were above 0.9, the correlations with ASI were slightly lower but still above 0.8. The positive correlation between anther quality and anther quantity showed that a normal fertile appearance of anthers was accompanied by a high number of anthers. Partial fertility was associated with protogyny, as indicated by the negative sign of the correlations between ASI and anther quality and quantity.

Linkage map and QTL results

The genetic map covered a total length of 1,322 cM. The average inter-marker distance was 4.0 cM with a maximum distance of 28.3 cM. 3.8% of the genotypic data were missing. Table 4 gives an overview over all detected QTL. In the joint analysis, the QTL for anther quality, anther quantity and ASI together explained 72.2, 70.2 and 62.3% of the phenotypic variance (adjusted R^2). In the individual trials, the adjusted R^2 values were in the same range. The QTL for anther quality and anther quantity usually explained more of the phenotypic variance than the QTL

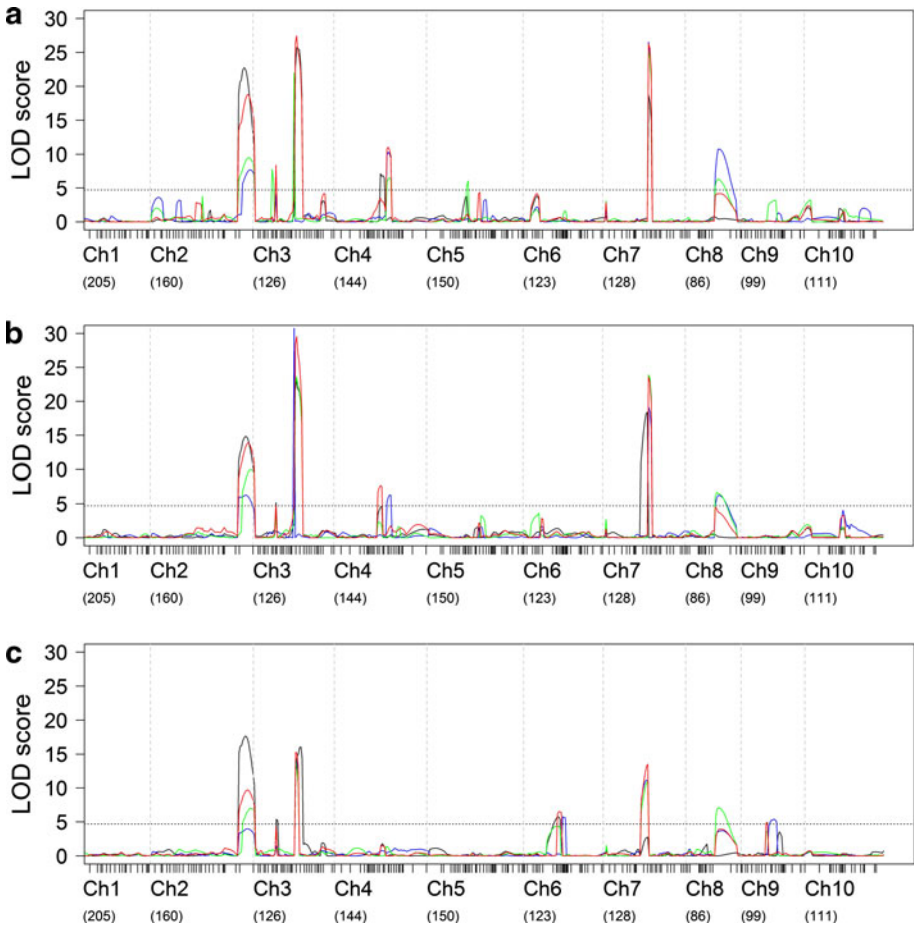

Fig. 4 LOD profiles for **a** anther quality, **b** anther quantity and **c** ASI at Cadenazzo (*black*), Delley (*blue*), Eschikon (*green*) and of the joint analysis (*red*). The *dashed horizontal lines* indicate the significance threshold (LOD = 4.7). The length (cM) of each chromosome (Ch) is given in *parentheses* (color figure online)

Table 3 Estimated additive effects and origin of the restorer (*Rf*) allele of the QTL for anther quality, anther quantity and the anthesis-silking interval (ASI) at Cadenazzo (CAD), Delley (DEL) and Eschikon (ESH) as well as in the joint analysis over three locations (Joint)

Trait	Bin	Additive effect				Origin of *Rf* allele
		CAD	DEL	ESH	Joint	
Anther quality	2.09	1.1	0.5	0.6	0.8	B37
	3.05	−0.6	–	−0.5	−0.5	K55
	3.06	−1.3	−0.8	−0.8	−1.1	K55
	4.06	−0.5	−0.5	−0.5	−0.5	K55
	5.03	–	–	−0.4	–	K55
	7.03	0.9	0.8	1.0	0.9	B37
	8.06	–	−0.5	−0.5	–	K55
Anther quantity	2.09	1.1	0.7	0.9	1.0	B37
	3.05	–	–	–	−0.6	K55
	3.06	−1.5	−1.6	−1.2	−1.6	K55
	4.06	–	−0.7	–	−0.6	K55
	7.03	1.2	1.3	1.3	1.3	B37
	8.06	–	0.7	0.7	–	K55
ASI	2.09	4.4	–	2.7	2.9	B37
	3.05	−2.6	–	–	–	K55
	3.06	−4.7	−4.2	−3.2	−3.7	K55
	6.04	2.1	2.3	–	2.1	B37
	7.03	–	3.3	2.7	3.2	B37
	8.06	–	–	−2.1	–	K55
	9.03	–	−2.4	–	−2.1	K55

for ASI (data not shown). The QTL analysis revealed three major QTL regions in the chromosomal bins 2.09, 3.06 and 7.03 (Fig. 4). In these genetic regions, QTL collocated, with a few exceptions, across all three fertility-related traits and all three environments. In most cases, they explained more than 10% of the phenotypic variance (npartR^2) and exceeded the LOD threshold of 4.7 by far. LOD scores as high as 30 suggest that partial restoration was inherited oligogenically. The restorer alleles of the QTL in bins 2.09 and 7.03 did not originate from the restorer parent K55 but from the maternal parent B37. The direction of effects was consistent across traits and environments (Table 3). The size of the additive effects indicates that the combination of partial restorer alleles at different loci can lead to nearly complete restoration of fertility even in the absence of the restoring *Rf4* allele. This conclusion is supported by the most fertile F_2BC_1 families, which putatively carried the restoring alleles at all or several major QTL, as opposed to the least fertile families, which putatively carried the non-restoring alleles.

As well as the three major QTL regions, other QTL for anther quality, anther quantity and the ASI were revealed on chromosomes 3, 4, 5, 6, 8 and 9 (Fig. 4). These QTL are considered to be minor QTL, because their effects usually explained less than 10% of the phenotypic variance. Although the detection of minor QTL was less consistent across traits and environments than that of the major QTL, clustering was observed in bins 3.05, 4.06, 4.07 and 8.06. However, QTL on chromosome 8 should be regarded with caution, because the fixation of the *rf4* allele might bias the QTL result. The restorer alleles of all the minor QTL regions originated from the restorer parent K55, except that in bin 6.04, where the maternal parent carried the fertility-enhancing allele (Table 3).

Minor QTL for anther quality were mapped to bins 3.05, 4.06 and 8.06 in the joint analysis and, with a few exceptions, in the individual trials as well. QTL for anther quality found in individual experiments but not in the joint analysis were located in bin 4.07 (DEL, ESH) and bin 5.03 (ESH). The latter QTL was specific for anther quality. QTL for anther quantity were detected in the same regions as for anther quality, except in bin 5.03. Bins 3.05 and 4.06 harbored QTL only in the joint analysis, whereas QTL in bin 8.06 were found at DEL and ESH but not in the joint analysis. The QTL for ASI differed in part from the QTL regions found for anther quality and anther quantity. No QTL were detected on chromosome 4, and QTL in bins 6.04 and 9.03 were significant for ASI only (DEL and joint analysis). QTL in bins 3.05 (CAD) and 8.06 (ESH) collocated with QTL found for anther quality and anther quantity.

The LOD support intervals for most major and minor QTL ranged from 8 to 18 cM around the respective peak. However, the support intervals for the QTL in bin 8.06 were larger, covering 22–28 cM. Numerous digenic interactions among QTL were found throughout the genome, but all of them exceeded the LOD threshold only marginally, and the interactions across traits and environments were inconsistent. Therefore, digenic interactions were not considered. Significant QTL × environment interactions were detected for anther quality only. The interactions were significant for the QTL in bins 3.06, 7.03 and 8.06, although these QTL were detected consistently in most of the individual trials as well as in the joint analysis. The frequencies of QTL detection, revealed by cross-validation, usually mirrored the pattern of LOD curves and confirmed the consistency of QTL detection across environments and fertility-related traits. QTL frequencies were higher for anther quality and anther quantity than for ASI. The highest QTL frequencies were found for the major QTL in bin 7.03 (25–93%, average 67%), whereas the frequencies were lower in the other two major QTL regions (bin 2.09: 15–34%, average 25%; bin 3.06: 23–69%, average 40%). The QTL frequencies of minor QTL ranged from 10 to 30% in most cases; however, QTL in bin 3.05 were detected with a frequencies up to 69% (average 44%).

Table 4 QTL for anther quality, anther quantity and the anthesis-silking interval (ASI) at Cadenazzo (CAD), Delley (DEL) and Eschikon (ESH) as well as in the joint analysis (Joint): chromosomal bin, genetic position, LOD support interval (SI interval), approximate genomic position projected on the IBM neighbors 2008 reference map (peak IBM), LOD score and normed partial R^2 (npartR^2)

Trait	Location	Bin	Peak (cM)	SI interval (cM)	Peak IBM (cM)	LOD	NpartR^2 (%)
Anther quality	CAD	2.09	142	130–158	623	22.7	16.5
		3.05	32	28–36	342	8.3	6.7
		3.06	64	60–76	515	25.7	20.1
		4.06	68	64–76	365	7.2	6.3
		7.03	64	60–72	298	18.7	17.1
	DEL	2.09	152	136–158	669	7.7	5.6
		3.06	60	56–64	494	16.1	22.4
		4.07	80	76–88	440	10.3	6.8
		7.03	64	60–72	298	26.6	22.0
		8.06	44	36–64	432	10.8	10.7
	ESH	2.09	150	130–158	661	10.0	5.8
		3.05	26	22–30	310	8.0	6.2
		3.06	60	56–64	494	21.5	15.9
		4.07	82	74–86	446	7.4	5.1
		5.03	60	56–64	189	5.8	6.0
		7.03	64	60–72	298	27.1	20.6
		8.06	42	34–62	430	7.9	8.9
	Joint	2.09	148	132–158	651	19.8	11.2
		3.05	32	28–36	343	8.7	6.6
		3.06	64	60–76	515	30.1	18.2
		4.06	68	60–72	365	12.1	7.0
		7.03	64	60–72	298	26.9	21.7
		8.06	40	36–64	388	5.9	7.5
Anther quantity	CAD	2.09	146	130–158	643	14.8	15.1
		3.06	64	60–76	515	23.1	30.0
		7.03	62	50–66	298	18.4	17.0
	DEL	2.09	146	132–158	643	6.3	6.8
		3.06	60	56–64	494	30.8	28.0
		4.07	82	76–88	446	6.3	6.3
		7.03	64	60–72	298	19.2	21.2
		8.06	42	34–66	432	6.3	7.8
	ESH	2.09	152	136–158	669	10.0	7.1
		3.06	64	60–76	515	23.7	26.8
		7.03	64	60–72	298	23.9	22.8
		8.06	40	34–62	388	6.7	7.2
	Joint	2.09	148	132–158	651	13.9	10.7
		3.05	32	28–36	343	4.8	3.7
		3.06	64	60–76	515	29.6	25.1
		4.06	68	60–72	365	7.7	6.6
		7.03	64	60–72	298	23.6	24.1

Table 4 continued

Trait	Location	Bin	Peak (cM)	SI interval (cM)	Peak IBM (cM)	LOD	NpartR^2 (%)
ASI	CAD	2.09	144	132–158	634	17.7	20.7
		3.05	32	30–38	342	5.4	9.6
		3.06	70	58–74	566	16.1	16.5
	DEL	3.06	62	58–70	498	14.1	23.0
		6.04	54	50–62	230	5.8	7.3
		7.03	60	48–64	295	11.2	14.8
		9.03	40	28–48	292	5.4	7.5
	ESH	2.09	152	136–158	651	7.0	7.8
		3.06	62	58–70	498	14.1	19.6
		7.03	62	50–66	298	10.9	15.1
		8.06	42	34–62	430	7.1	8.1
	Joint	2.09	148	132–158	651	9.7	10.9
		3.06	62	58–70	498	15.3	22.8
		6.04	48	44–56	210	6.5	6.9
		7.03	62	50–66	298	13.5	14.1
		9.03	30	26–34	256	4.9	7.6

Discussion

Weak effect of the environment on partial restoration

The effect of environment on partial restoration was weak. Accordingly, heritabilities for anther quality, anther quantity and ASI were high. This reflects the accuracy and reproducibility of the experimental conditions and the scoring method used to evaluate partial restoration in the field. Compared to the fertility-related traits, the heritability of plant height was lower, both in the joint analysis across environments as well as in the individual trials. The discrepancy indicates that growth conditions differed in the fields but did not impact the restoration of fertility. This corroborates the results of Kaul (1988), who found that edaphic factors are less important for the expression of male fertility than climatic factors, particularly temperature and photoperiod. The high heritabilities of fertility-related traits may be due to the relatively cool and moist climatic conditions in all the environments, because these conditions are assumed to be conducive to the expression of male fertility (Duvick 1965; Tracy et al. 1991). Therefore, Switzerland might be a suitable place to detect QTL involved in partial restoration and to select male-sterile inbred lines. However, to quantify the effect of the environment on the expression of QTL, locations with a continental climate and at a different latitude should be included.

There are no comparable values for the heritability of partial restoration reported in the literature. Most studies on restoration of CMS report a strong environmental impact (Tracy et al. 1991; Weider et al. 2009). In a study about the partial restoration of C-CMS in A632 background, Tracy et al. (1991) observed large differences among years, which made it impossible to infer the number of genes involved. In contrast, a study about C-CMS with a wide range of germplasm grown at two locations and in two seasons showed that the effect of environments was of minor importance compared to the nuclear background of CMS plants (Vidakovic 1988). Such inconsistent results concerning the effect of environment probably result from the specific choice of germplasm and test locations.

Partial restoration is inherited like an oligogenic trait

Major QTL with very high LOD scores and stable expression across environments and fertility-related traits suggest that partial restoration was inherited oligogenically. Therefore, a marker-assisted selection of the QTL in bins 2.09, 3.06 and 7.03 seems feasible if their importance for different germplasm and in a broader range of environments can be proven. The existence of major QTL corroborates the field observations of Gontarovskii (1974) and Tracy et al. (1991), who reported that paired cross selection (Jones and Manglesdorf 1951) is often successful for reducing partial restoration in C- and T-CMS inbred lines over a few generations. It is assumed that this method will be successful only when few major genes govern the trait. As well as the major QTL, there were numerous minor QTL, which were expressed inconsistently across environments. Such loci are probably responsible for the differences in partial restoration in many studies. The lower correlation and minor differences in the patterns of QTL detection between ASI versus anther quality and anther

quantity (Table 3; Fig. 4) suggest that some of the variation in ASI were due to genetic effects unassociated with restoration of fertility, e.g. a heritable difference between the ASI of the paternal inbred lines, which would be measurable when both lines are in normal cytoplasm.

Interactions between restorer QTL were not found in this study, although interacting restorer genes have been reported earlier in other crosses, where restoration of fertility might be controlled by different loci (Vidakovic 1988; Hu et al. 2006). Interactions among *Rf4* and partial restorer QTL could not be tested in this experiment due to the fixation of the *rf4* allele. Interactions with *Rf4* should, therefore, be addressed in a mapping population comprising *Rf4rf4* individuals.

In a marker-assisted selection program against partial restorer genes, care needs to be taken not to eliminate alleles that might be necessary for the full restoration of the hybrid. Such a situation can arise when the restoration depends on the complementary interaction of maternal and paternal factors and should be avoided by continuous testcrossing of the maternal by the restorer parent.

Major QTL collocate with other restorer genes of maize

All three major QTL regions found in this study map near other restorer genes of maize. The QTL region in bin 2.09 collocates with a cluster of multiple restorer genes for S-CMS, including the major restorer gene *Rf3* (Gabay-Laughnan et al. 2004), and *Rf8* and *Rf**, which restore T-CMS in the presence of *Rf2* (Dill et al. 1997). The QTL in bins 3.05 and 3.06 are linked to *Rf1* of T-CMS in bin 3.04. Sisco (1991) assumed restorer genes for C-CMS in this region, because the genomic area around the major restorer gene *Rf4* in bin 8.00 shows collinearity of the homologous sequences, as revealed by hybridization of RFLP probes (Gaut 2001). Annotating and comparing the duplicated genome regions could, therefore, lead to the identification of both *Rf4* and the genes underlying the QTL in bins 3.05 and 3.06. The QTL region in bin 7.03 maps near *Rf-I*, an inhibitor of *Rf5* of C-CMS and may be identical to *Rf7*, which also acts as a partial restorer gene (Qin et al. 1990; Hu et al. 2006).

The clustering of restorer genes in maize corresponds to the complex genomic structure of restorer loci in other plants such as petunia, radish and rice, where restorer genes are found near highly homologous genes of the pentatricopeptide-repeat (PPR) family (Bentolila et al. 2002; Desloire et al. 2003; Wang et al. 2008). Furthermore, clusters of uncharacterized restorer genes were found in cotton (Zhang and Stewart 2001) and rapeseed (Li et al. 1998). Such clusters might consist of paralogous genes, which probably arise from repeated duplications through unequal crossing over (Touzet and Budar 2004). This process might facilitate the evolution of restorer genes in response to the spread of CMS in a hermaphroditic population of plants. Therefore, the high number of partial restorer genes in the maize genome might witness ancient constitutions of the mitochondrial genome, or these genes might have arisen as defective duplicates of existing restorer genes. Although linkage does not imply similarity of sequences (Osbourn 2010), linked restorer genes may have a common evolutionary origin and, thus, possibly a common mode of action. It has been hypothesized that paralogous restorer genes can diverge to restore different CMS systems (Bentolila et al. 2002; Touzet and Budar 2004; Wang et al. 2006). This hypothesis could be tested by dissecting the genetic structure of the long arm of chromosome 2, which contains restorer genes for all three major CMS systems in maize (T, S and C).

The maternal parent is a source of restorer alleles

The maternal parent was clearly involved in the partial restoration of the mapping population, supporting previous results with different populations (Vidakovic 1988; Sotchenko et al. 2007). It is tempting to speculate that the QTL in bins 2.09, 6.04 and 7.03 were responsible for the partial restoration of B37C. Whereas reversions to fertility in B37C have not been reported by the Maize Genetics Stock Center, some anthers filled with pollen were found occasionally under field conditions in Serbia and Missouri (USA) (M. Vidakovic, K. Newton, pers. comm., 2009). The expression of partial restorer alleles in B37C is probably usually suppressed at its site of origin, but favored by the climatic conditions in Switzerland.

Conclusions

Partial restoration of male fertility was inherited like an oligogenic trait. Therefore, a marker-assisted counterselection of major QTL in bins 2.09, 3.06 and 7.03 is promising, but the importance of these loci must be verified with other germplasm and under different environmental conditions. Elucidating the genetic structure of clusters of restorer genes could improve our understanding of the evolution and functioning of restorer genes. In this respect, the long arm of chromosome 2 is particularly interesting, because it contains restorer genes for all three major CMS systems in maize.

Acknowledgments We are grateful to Karl-Heinz Camp and Delley Seeds and Plants Ltd (Delley, Switzerland), Mario Bertossa and Agroscope Changins-Wädenswil in Cadenazzo (Switzerland) and Ernst Weiss for their support of the field trials. Agroscope Reckenholz-Tänikon (Zurich, Switzerland) provided access to JoinMap 4.0.

We further thank Marianne Wettstein for her excellent technical assistance and Angela Steinauer, Adrian Ramseyer and Marylène Maillard for their assistance in the collection of data.

References

Bentolila S, Alfonso AA, Hanson MR (2002) A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc Natl Acad Sci USA 99:10887–10892

Butler DG, Cullis BR, Gilmour AR, Gogel BJ (2007) Analysis of mixed models for S language environments. ASReml-R reference manual. Release 2 (2007). Queensland Department of Primary Industries and Fisheries, GPO Box 46, Brisbane, QLD, 4001, Australia

Churchill GA, Doerge RW (1996) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Desloire S, Gherbi H, Laloui W, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. EMBO Rep 4:588–594

Dill CL, Wise RP, Schnable PS (1997) *Rf8* and *Rf** mediate unique T-*urf13*-transcript accumulation, revealing a conserved motif associated with RNA processing and restoration of pollen fertility in T-cytoplasm maize. Genetics 147:1367–1379

Duvick DN (1956) Allelism and comparative genetics of fertility restoration of cytoplasmically pollen sterile maize. Genetics 41:544–565

Duvick DN (1965) Cytoplasmic pollen sterility in corn. Adv Genet 13:2–52

Fan J, Oliphant A, Shen R, Kermani B, Garcia F, Gunderson K, Hansen M, Steemers F, Butler S, Deloukas P, Galver L, Hunt S, McBride C, Bibikova M, Rubano T, Chen J, Wickham E, Doucet D, Chang W, Campbell D, Zhang B, Krugylak S, Bentley D, Haas J, Rigault P, Zhou L, Stuelpnagel J, Chee M (2003) Highly parallel SNP genotyping. Cold Spring Harb Symp Quant Biol 68:6978

Gabay-Laughnan S, Chase CD, Ortega VM, Zhao LM (2004) Molecular-genetic characterization of CMS-S *restorer-of-fertility* alleles identified in Mexican maize and teosinte. Genetics 166:959–970

Gaut BS (2001) Patterns of chromosomal duplication in maize and their implications for comparative maps of the grasses. Genome Res 11:55–66

Gontarovskii V (1974) Role of supplementary genes in control of Texas type of CMS in maize. Genetika 10:15–24

Has V (2002) Evaluation of some maize inbred lines on fertility restoration patterns of male-sterile cytoplasms. Romanian Agric Res 17:1–7

Hospital F, Moreau L, Lacoudre F, Charcosset A, Gallais A (1997) More on the efficiency of marker-assisted selection. Theor Appl Genet 95:1181–1189

Hu Y, Tang J, Yang H, Xie H, Lu X, Niu J, Chen W (2006) Identification and mapping of *Rf-I* an inhibitor of the *Rf5* restorer gene for Cms-C in maize (*Zea mays* L.). Theor Appl Genet 113:357–360

IUSS Working Group WRB (2007) World Reference Base for Soil Resources 2006, first update 2007. World Soil Resources Reports No. 103. FAO, Rome

Jones DF, Manglesdorf PC (1951) The production of hybrid corn seed without detasseling. Conn Agric Exper Sta Bull 550

Kaul M (1988) Male sterility in higher plants. Springer, Berlin

Kheyr-Pour A, Gracen VE, Everett HL (1981) Genetics of fertility restoration in the C-group of cytoplasmic male-sterility in maize. Genetics 98:379–388

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugenics 12:172–175

Li HH, Ye GY, Wang JK (2007) A modified algorithm for the improvement of composite interval mapping. Genetics 175:361–374

Li XQ, Jean M, Landry BS, Brown GG (1998) Restorer genes for different forms of *Brassica* cytoplasmic male sterility map to a single nuclear locus that modifies transcripts of several mitochondrial genes. Proc Natl Acad Sci USA 95:10032–10037

Munsch A, Stamp P, Christov NK, Foueillassar XM, Hüsken A, Camp KH, Weider C (2009) Grain yield increase and pollen containment by Plus-Hybrids could improve acceptance of transgenic maize. Crop Sci 50:909–919

Osbourn A (2010) Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. Trends Genet 26:449–457

Qin T, Xu M, Dun D (1990) Cytoplasmic male sterility: identification of the number of the restorer genes. MNL 64:124

R Development Core Team (2009) A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. http://www.R-project.org. Accessed 10 November 2010

Schaeffer (Polacco) M, Sanchez-Villeda H, Coe E (2008) IBM neighbors 2008 map. Available at http://www.maizegdb.org. Maize Genetics and Genomics Database. Accessed 10 November 2010

Sisco PH (1991) Duplications complicate genetic mapping of *Rf4*, a restorer for CMS-C cytoplasmic male sterility in corn. Crop Sci 31:1263–1266

Soller M, Beckmann JS (1990) Marker-based mapping of quantitative trait loci using replicated progenies. Theor Appl Genet 80:205–208

Sotchenko V, Gorbacheva A, Kosogorova N (2007) C-type cytoplasmic male sterility in corn. Russian Agric Sci 33:83–86

Touzet P, Budar F (2004) Unveiling the molecular arms race between two conflicting genomes in cytoplasmic male sterility?. Trends Plant Sci 9:568–570

Tracy WF, Everett HL, Gracen VE (1991) Inheritance, environmental effects, and partial male-fertility in C-type CMS in a maize inbred. J Hered 82:343–346

Utz H, Melchinger A (2000) PLABQTL: a computer program for statistical analysis of plant breeding experiments. Release version 1.1

Van Ooijen J (2006) JoinMap4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, The Netherlands

Vidakovic M (1988) Genetics of fertility restoration in cytoplasmic male-sterility of the c-type (cmsC) in maize (*Zea mays* L.). Maydica 33:51–64

Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, Long Y, Zhong Y, Liu Y (2006) Cytoplasmic male sterility of rice with Boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell 18:676–687

Wang ZW, Zhang YJ, Xiang CP, Mei SY, Zhou Y, Chen GP, Wang T (2008) A new fertility restorer locus linked closely to the *Rfo* locus for cytoplasmic male sterility in radish. Theor Appl Genet 117:313–320

Weider C, Stamp P, Christov N, Hüsken A, Foueillassar X, Camp KH, Munsch M (2009) Stability of cytoplasmic male sterility in maize under different environmental conditions. Crop Sci 49:77–84

Zeng ZB (1994) Precision mapping of quantitative trait loci. Genetics 136:1457–1468

Zhang JF, Stewart JM (2001) Inheritance and genetic relationships of the D8 and D2-2 restorer genes for cotton cytoplasmic male sterility. Crop Sci 41:289–294

Zhu S, Rossnagel BG, Kaeppler HF (2004) Genetic analysis of quantitative trait loci for groat protein and oil content in oat. Crop Sci 44:254–260