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Genetic approaches to dissect iron efficiency in maize

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Contents

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Abbreviations

DMA	deoxymugineic acid
DMAS	deoxymugineic acid synthase
Fe	iron
GO	gene ontology
GWAS	genome-wide association study
HapMap	haplotype map
HKA	Hudson Kreitman Aguadé
IBM	intermated B73 and Mo17
IRIL	intermated recombinant inbred line
LD	linkage disequilibrium
MTK	methylthioribose kinase
MTP	metal transporter protein
NA	nicotianamine
NAM	nested association mapping
NAS	nicotianamine synthase
NAAT	nicotianamine aminotransferase
NRAMP	natural resistance associated macrophage protein
pH	power of hydrogen
PHT	phosphorous transporter
π	nucleotide diversity
PS	phytosiderophore
QIR	QTL isogenic recombinant
QTL	quantitative trait locus
RNAi	ribonucleic acid interference
SNP	single-nucleotide polymorphism
SSR	simple sequence repeat
TILLING	targeting-induced local lesions in genomes
TOM1	transporter of mugineic acid 1
UniProt	universal protein
VIT1	vacuolar iron transporter 1
YS1	yellow stripe 1

1. General Introduction

Iron (Fe) deficiency in maize (*Zea mays* subsp. *mays*) is mostly caused by growth on calcareous or alkaline soils that are characterized by a high pH. Under such conditions, Fe precipitates in form of hydroxides (Marschner, 1995) and consequently, Fe has a low availability for plants (Römheld and Marschner, 1983). As a consequence, Fe-deficiency occurs and induces leaf chlorosis. Leaf chlorosis leads to reduced Fe content in harvest products, and decreases the yield (Curie and Briat, 2003; Godsey et al., 2003). The biological and physiological comprehension of the mechanisms involved in Fe uptake and homeostasis within the plant are afferent for Fe efficiency dissection in maize. Moreover, consideration of leaf mineral nutrient concentrations as well as physiological and morphological aspects in a controlled environment will aid to deduce the phenotype development in maize. The general goal of my studies is to provide both genes and genetic markers for functional analyses and marker-assisted breeding programs dealing with the improvement of Fe-efficiency in maize.

Iron uptake and homeostasis

Graminaceous plant species like maize acquire Fe by the so-called strategy II mechanism, which include the release of phytosiderophores, acting as high-affinity hexadentate chelators for ferric Fe, and an elevated expression of transport systems for Fe(III)-phytosiderophores at the root plasma membrane (Winkelmann et al., 1987). However, relative to barley, maize releases

approximately fivefold lower amounts of phytosiderophores (Marschner, 1995). This may explain at least partially the high susceptibility of maize to Fe deficiency-induced chlorosis. In maize, characterization of intraspecific variation of tolerance to Fe-deficiency in the form of induced chlorosis, chlorosis tolerance and its genetic differences or other Fe-efficiency traits across a larger population of maize genotypes, have not yet been reported.

Previous studies examining other graminaceous plant species have characterized various essential components and genes involved in Fe-efficiency, i.e. the ability of plants to produce less chlorotic leaves or higher biomass or grain yield. For instance, one aspect was sulfur uptake that is necessary for synthesis of methionine (Hopkins et al., 2004). This holds particularly interest for genes involved in phytosiderophore biosynthesis, in which nicotianamine synthase (NAS) is required for the conjugation of three *S*-adenosyl-methionine residues to produce nicotianamine (NA) (Higuchi et al., 1999). NA is then subjected to subsequent amino transfer by nicotianamine aminotransferase (NAAT) (Inoue et al., 2008) and a reduction step by deoxy-mugineic acid synthase (DMAS) to yield deoxy-mugineic acid (DMA), which is the only phytosiderophore species being released by maize (Bashir et al., 2006). Subsequently, DMA is released by the transporter of mugineic acid 1 (TOM1) which is localized at the root plasma membrane (Nozoye et al., 2011). Following metal chelation in the rhizosphere, the uptake of Fe(III)-phytosiderophores into root cells is mediated by membrane proteins of the YS1 family that possess a particularly high affinity for phytosiderophore-chelated ferric Fe (Curie et al., 2001; Schaaf et al., 2004). Inside root cells, ferric Fe may be reduced and exchange chelated to nicotianamine (von Wirén et al., 1999) and further transported radially for xylem loading. In seeds and young seedlings, vacuolar loading and unloading are critical for Fe-efficiency. In this respect, Fe loading of the vacuole by the vacuolar iron transporter 1 (VIT1) (Kim et al., 2006), remobilization therefrom by the natural resistance associated macrophage protein (NRAMP3 and 4) are essential processes for early seedling development under Fe-limiting growth conditions, as demonstrated in Arabidopsis (Lanquar et al., 2005).

Population genetics analyses to detect signatures of selection

Quantitative trait loci (QTL) and association analyses require phenotypic variation and polymorphic markers for the identification of genome regions contributing to phenotypic variance. If variation at candidate genes is limited because of selection during domestication and (or) crop improvement, the analyses would fail to identify these genes, contributing to trait variation (Yamasaki et al., 2005; Flint-Garcia et al., 2009). Therefore, comparison of genes with low diversity in modern maize varieties relative to their progenitors may facilitate the discovery of genes important to crop improvement (Wright et al., 2005; Flint-Garcia et al., 2009).

Maize was domesticated from teosinte (*Zea mays* subsp. *parviglumis*) 6,000 − 10,000 years ago (Matsuoka et al., 2002). The major traits that were targeted during maize domestication include the facilitation of harvest and adaptation to new environmental conditions (Buckler et al., 2001). In addition, yield and quality traits received primary attention (Flint-Garcia et al., 2009). Therefore, Fe-efficiency in maize might has been the target of selection during domestication. However, despite the essential role of Fe in plants and the fact that severe Fe-deficiency symptoms of maize occur on soils with high pH and its negative influence on plant fitness (Kobayashi et al., 2009), it remains unknown whether Fe-efficiency is an adaptive trait in maize.

QTL mapping to dissect genetic architecture in the IBM population

Despite the comprehensive knowledge on the functional aspects of Fe uptake and Fe homeostasis, to the best of our knowledge, no earlier study examined the natural variation of Fe-efficiency and mineral homeostasis in maize. In fact, this information will be valuable for the development of maize cultivars adapted to calcareous soils by classical plant breeding methods. Furthermore, when such analyses are linked to molecular marker information they have the potential to identify genes mechanistically involved in the trait of interest that have not been identified in classical functional/forward genetics studies. This is due to the fact that in contrast to mutant screens which consider one gene in one genetic background (Kobayashi and Koyama, 2002), analyses of natural variation study empowers the detection of multiple genes in complex genetic backgrounds (Salt et al., 2008).

Identification of QTL provides information on the chromosomal locations contributing to the quantitative variation of complex traits occurring within segregating populations (Mitchell-Olds and Pedersen, 1998; Zhang et al., 2010; Buescher et al., 2010). For maize, the intermated B73 x Mo17 (IBM) segregating population is a genetic resource with a high genetic map resolution (Lee et al., 2002). Further benefit of QTL mapping compared to mutant screens is the possibility to detect multiple genes which may be associated with the phenotypic trait (*cf.* Kobayashi and Koyama, 2002). In addition to the mapping of QTLs, the combination with expression studies of positional candidate genes have the potential to improve our understanding of the QTL of interest, the underlying genes, and in consequence the functional mechanism affecting the investigated trait.

Association mapping to dissect genetic architecture in the maize association population

Linkage mapping is depending on polymorphisms between both parental lines and allele recombination of their progenies. Hitherto, allele fixation or limited recombination events are the bottleneck in QTL detection (Flint-Garcia et al., 2003, 2009). Association mapping promises to overcome the limitations of low allele diversity and absence of recombination events (Flint-Garcia et al., 2003), which causes poor resolution in detecting QTL by linkage mapping (Stich and Melchinger, 2009).

The publicly available maize association mapping that was used for association mapping in former studies promises to provide a finer resolution of alleles (Flint-Garcia et al., 2005; Cook et al., 2012; Larsson et al., 2013) than standard bi-parental crosses (Oraguzie and Wilcox, 2007). Unlike in the bi-parental cross population, the association mapping population might contain population stratification and unequal allele distribution within these groups (Flint-Garcia et al., 2003). Therefore, knowledge about population structure is required to reduce the number of spurious associations (Pritchard et al., 2000; Liu et al., 2003; Flint-Garcia et al., 2005; Stich et al., 2005). Since the confidence intervals of QTLs capture several centimorgans that comprising hundreds of genes association mapping might provide the location of the gene causing phenotypic variation of the representative trait. In addition to that, genome-wide association mapping has the potential and the benefit of a high mapping resolution and thus, provides new genetic insights on Fe-efficiency. To our knowledge, no association study has been conducted to dissect Fe-efficiency in maize.

Objectives

The general goal of this thesis was to dissect genetically the trait Fe-efficiency and provide both genes and genetic markers for functional analyses and marker-assisted breeding programs in maize. In particular, the objectives were to

1. describe patterns of sequence variation of 14 genes involved in mobilization, uptake, and transport of Fe, and to determine if these genes were targets of selection during domestication;

2. determine the natural genetic variation of morphological/physiological traits in response to deficient and sufficient Fe regimes in the maize intermated B73 x Mo17 (IBM) population and to identify QTLs associated with these traits;

3. analyze Fe-dependent expression levels of genes known to be involved in Fe homeostasis as well as positional candidate genes from QTL analysis;

4. determine the natural genetic variation of mineral nutrient concentration in maize leaves of the intermated B73 x Mo17 (IBM) population;

5. evaluate the influence of different iron regimes on correlations among mineral nutrient concentrations;

6. identify QTLs which contribute to the mineral nutrient concentration difference;

7. identify polymorphisms affecting morphological/physiological traits by association analyses; and

8. fine map QTL confidence intervals by using association genetics.

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An analysis of selection on candidate genes for regulation, mobilization, uptake, and transport of iron in maize

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Abstract

Sparse iron (Fe) availability, which frequently occurs in soils with alkaline pH, can lead to leaf chlorosis, a reduced Fe content in harvest products, and yield reduction in maize. The objectives of this study were (i) to describe patterns of sequence variation of 14 candidate genes for mobilization, uptake, and transport of Fe in maize, as well as regulatory function on these processes, (ii) to examine whether Fe-efficiency is an adaptive trait by determining if these genes were targets of selection during domestication, and (iii) to test if the allele distribution at these candidate genes is different for the different sub-populations of maize. The nucleotide diversity of *Mtk* was reduced by

78% in maize compared to teosinte. The results of our study revealed for the genes *Naat1, Nas1, Nramp3, Mtk*, and *Ys1* a selective sweep, which suggests that these genes might be important for the fast adaptation of maize to new environments with different Fe availabilities.

The genetic basis of natural variation for iron homeostasis in the maize IBM population

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Abstract

Iron (Fe) deficiency symptoms in maize (*Zea mays* subsp. *mays*) express as leaf chlorosis, growth retardation, as well as yield reduction and are typically observed when plants grow in calcareous soils at alkaline pH. To improve our understanding of genotypical variability in the tolerance to Fe deficiency-induced chlorosis, the objectives of this study were to (i) determine the natural genetic variation of traits related to Fe homeostasis in the maize

intermated B73 x Mo17 (IBM) population, (ii) to identify quantitative trait loci (QTLs) for these traits, and (iii) to analyze expression levels of genes known to be involved in Fe homeostasis as well as of candidate genes obtained from the QTL analysis. In hydroponically-grown maize, a total of 47 and 39 QTLs were detected for the traits recorded under limited and adequate supply of Fe, respectively. From the QTL results, we identified new putative candidate genes involved in Fe homeostasis under a deficient or adequate Fe nutritional status, like Ferredoxin class gene, putative ferredoxin *PETF*, metal tolerance protein *MTP4*, and *MTP8*. Furthermore, our expression analysis of candidate genes suggested the importance of *trans*-acting regulation for 2'-deoxymugineic acid synthase 1 (*DMAS1*), nicotianamine synthase (*NAS3, NAS1*), formate dehydrogenase 1 (*FDH1*), methylthioribose-1-phosphate isomerase (*IDI2*), aspartate/tyrosine/aromatic aminotransferase (*IDI4*), and methylthioribose kinase (*MTK*).

Natural variation of mineral element homeostasis in maize examined by QTL mapping

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Natural variation of mineral nutrient homeostasis in maize examined by QTL mapping

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ABSTRACT

Mineral nutrients are essential for plant development, yield formation and quality traits in crops. Keeping a balanced nutrition is complicated by the fact that the deficiency in one mineral element often perturbs the homeostasis of others. As little is known on how this balance is kept at the genome level, we investigated the genotypic variability of mineral element homeostasis in maize plants subjected to Fe deficiency. The objectives of this study were to (i) determine the natural genetic variation of mineral element concentrations in the leaves of the maize intermated B73 x Mo17 (IBM) population, (ii) evaluate the influence of different iron (Fe) regimes on mineral element concentrations, (iii) determine the correlation between mineral elements and physiological as well as morphological traits, and (iv) identify QTLs which contribute to variations in mineral element concentrations. Under deficient Fe supply, mineral element concentrations in maize leaves increased significantly for all mineral elements, except for sodium (Na). In total, 44 and 13 QTLs were detected for variations in mineral element concentrations evaluated under deficient and sufficient Fe supplies, respectively. The QTL confidence intervals comprised genes involved in mineral element homeostasis under varying Fe nutrition, such as genes that sequester Cd in vacuoles (*HMA3*), transport Zn and Fe in root cells (*ZIP10, NRAMP2*), protect cells against oxidative stress (glutaredoxin), regulate protein activities (PP2C), or prevent deleterious accumulation and interaction of specific nutrients within cells (*PHT1;5, ZIP4*).

The uptake of mineral elements by roots requires specific transport systems at the root plasma membrane and in some cases also specialized mobilization strategies for the acquisition of sparingly soluble elements from the rhizosphere. However, many nutrient transporters show broad substrate specificities leading to an excess uptake of undesired elements which may cause imbalances of mineral elements in plants tissues (Fodor, 2006). To counteract nutrient imbalances plants have evolved mechanisms to safeguard mineral element homeostasis in individual plant tissues to prevent antagonistic interactions among nutrients, such as frequently reported for Fe and P (Zheng et al., 2009; Baxter et al., 2012; Shi et al., 2012).

Iron (Fe) belongs to those essential elements which readily precipitate in soils whenever the soil pH becomes neutral or alkaline, is well aerated or contains little organic matter (Marschner, 2012). Therefore, plants need to solubilize sparingly soluble Fe in the rhizosphere, which is mediated by the secretion of mugineic acid-type or coumarin-type siderophores into the rhizosphere (Kobayashi and Nishizawa, 2012; Schmid et al., 2014). Due to the low substrate specificity of the transport systems for ferrous or chelated Fe, which are IRT1- or YS1/YSL-type transporters, respectively, other, undesired heavy metals as taken up as well (Giehl et al., 2009). For instance, maize plants grown under Fe deficiency accumulated two times more of the toxic heavy metal cadmium (Cd) in roots and shoots than under an Fe-sufficient regime (Meda et al., 2007). The accumulation of Cd in the plant can in turn disturb intracellular Fe signaling pathways (Clemens, 2006), decrease photosynthetic activity and inhibit plant growth (Deckert, 2005). Besides toxic minerals, essential minerals like phosphorous (P) accumulate in the plant during Fe deficiency (Zheng et al., 2009). The experiments of Hansen et al. (2006); Zheng et al. (2009) indicated that the accumulation of P might lead to antagonistic interactions with Fe, which promotes Fe-deficiency symptoms under limited Fe supply and consequently causes crop yield reduction and quality loss. Likewise, Kanai et al. (2009) showed that Fe deficiency enhanced the accumulation of Zn in leaves causing severe growth reduction in maize. However, in contrast to the before mentioned elements, the accumulation of other essential mineral nutrients does not lead necessarily to more severe Fe deficiency-induced chlorosis. This was shown for potassium (K) and sulfur (S) accumulation that play an essential role in the production and release of phytosiderophores (Mori and Nishizawa, 1987). Moreover, a higher concentrations of both nutrients was shown to improve Fe acquisition under low Fe conditions (Mori and Nishizawa, 1987; Astolfi et al., 2003).

Accumulation mechanisms of mineral elements in

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Table 1 Broad sense heritability on an entry mean basis (H^2) for the concentrations of 16 mineral nutrients and 13 morphological and physiological traits recorded for two iron regimes (Deficient and Sufficient). For details, see materials and methods.

			H^2	
Trait	Abbreviation	Unit	Deficient	Sufficient
Aluminum	Al	μg/g DW	0.58	0.58
Boron	B	μg/g DW	0.73	0.52
Cadmium	Cd	μg/g DW	0.76	0.86
Calcium	Ca	mg/g DW	0.70	0.72
Cobalt	Co	μg/g DW	0.50	0.44
Copper	Cu	μg/g DW	0.77	0.76
Iron	Fe	μg/g DW	0.65	0.64
Magnesium	Mg	mg/g DW	0.70	0.69
Manganese	Mn	μg/g DW	0.77	0.71
Molybdenum	Mo	μg/g DW	0.95	0.95
Nickel	Ni	μg/g DW	0.30	0.35
Phosphorus	P	mg/g DW	0.82	0.43
Potassium	K	mg/g DW	0.59	0.41
Sodium	Na	μg/g DW	0.57	0.55
Sulfur	S	mg/g DW	0.82	0.62
Zinc	Zn	μg/g DW	0.85	0.67
SPAD value at leaf 3[1]	SP3		0.80	0.67
SPAD value at leaf 4[1]	SP4		0.77	0.70
SPAD value at leaf 5[1]	SP5		0.80	0.80
SPAD value at leaf 6[1]	SP6		0.75	0.64
Root length[1]	RL	cm	0.51	0.42
Root weight[1]	RW	g	0.66	0.50
Shoot length[1]	SL	cm	0.35	0.28
Shoot dry weight[1]	SDW	g	0.58	0.38
Shoot water content[1]	H_2O	%	0.65	0.65
Ratio between shoot dry weight and shoot length[1]	SDW/SL	g/cm	0.53	0.41
Branching at the terminal 5 cm of root[1]	BTR		0.64	[2]
Lateral root formation[1]	LAT		0.55	0.58
Leaf necrosis[1]	NEC		0.44	0.59

[1] evaluated by Benke et al. (under review)

[2] no variation observed

maize leaves and their strengthening or mitigation of Fe-chlorosis under Fe deficiency is well known. However, attempts to characterize the effect of varying Fe concentrations in the rhizosphere on the natural variation of mineral nutrient concentrations were not reported for vegetative organs of maize.

In addition, analyses on natural allelic variation of mineral element homeostasis in Fe-deficient maize have, to the best of our knowledge, not been reported yet.

The breeding of Fe-inefficient crops, such as rice or maize, for improved tolerance to soils with low Fe availability will be facilitated not only by the identification of loci or genes which promote Fe acquisition but also of those maintaining mineral element homeostasis (*cf.* Baxter et al., 2012). A powerful method to reach this goal is the mapping of quantitative trait loci (QTL) (Mitchell-Olds and Pedersen, 1998; Buescher et al., 2010). For maize, the intermated B73 x Mo17 (IBM) segregating population is a publicly available resource allowing QTL detection with a high mapping resolution (Lee et al., 2002).

The objectives of this study were to (i) determine the natural genetic variation and identify QTL for mineral element concentrations in the leaves of the maize intermated B73 x Mo17 (IBM) population, (ii) evaluate the influence of different Fe regimes on mineral element concentrations, and (iii) determine the correlation between mineral elements and physiological as well as morphological traits.

MATERIALS AND METHODS

Plant material

The intermated recombinant inbred lines (IRILs) of the IBM population, which was derived from a cross of the maize inbreds B73 and Mo17 (Lee et al., 2002), was examined in the current study. Due to the unavailability of seeds for the IRILs MO040, MO043, MO048, MO057, MO062, MO063, MO076, MO079, and MO344, a total of 85 IRILs were evaluated in this study.

Culture conditions and measured mineral elements

Maize seeds were sterilized in a 3% NaClO solution for 3 minutes and then treated with 60°C hot water for 5 minutes. Afterwards, seeds were placed between two filter paper sheets moistened with saturated $CaSO_4$ solution for germination in the dark at room temperature.

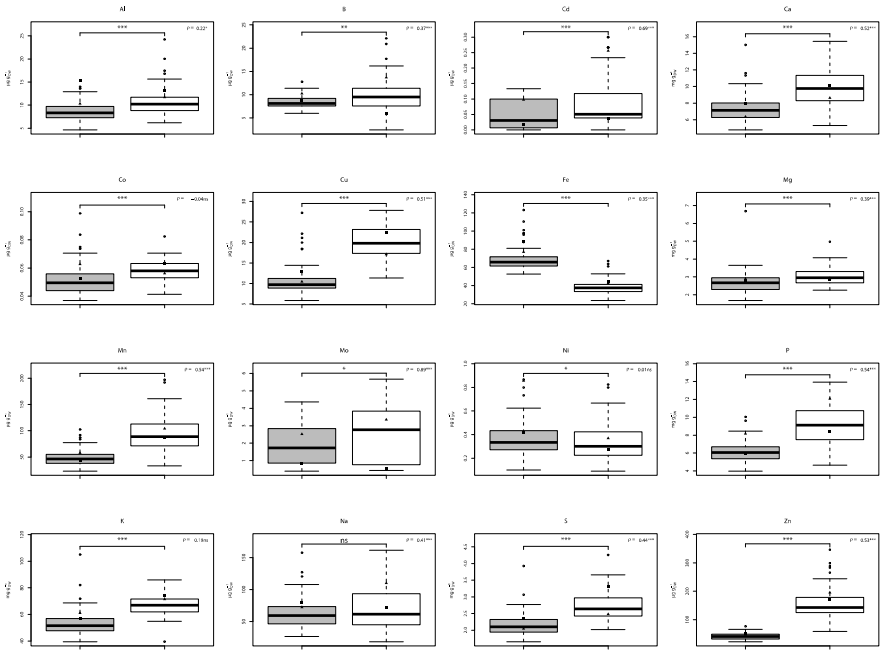

Fig. 1 Boxplot of the adjusted entry means (AEM) of the concentrations of 16 mineral nutrients for the 85 maize inbred lines of the maize IBM population measured at sufficient (grey) and deficient (white) iron regimes. The adjusted entry means of the parental inbreds B73 and Mo17 are represented by a square and a triangle, respectively. A *t*-test was used to examine the statistical significance of the mean difference for a trait measured at two different iron regimes across the population. Spearman rank correlation coefficient (ρ) was calculated between the AEM of both iron regimes. *, **, ***: P = 0.05, 0.01, and 0.001, respectively; ns, not significant.

After 6 days, the germinated seeds were transplanted to a continuously aerated nutrient solution with element concentrations as described by von Wirén et al. (1996). The plants were supplied with 100 μM Fe(III)-EDTA for 7 days. From day 14 to 28, plants were supplied with 10 (Fe deficient) or 300 (Fe sufficient) μM Fe(III)-EDTA. The nutrient solution was exchanged every third day.

Plants were cultivated from day 7 to day 28 in a growth chamber at a relative humidity of 60%, a light intensity of 170 μmol m^{-2} s^{-1} in the leaf canopy, and a day-night temperature regime of 16 h/24°C and 8 h/22°C, respectively.

Four plants of each IRIL were grown in one shaded 5 L pot. Two separate pots were used for the two examined Fe concentrations. All plots were arranged in a growth chamber following a split-plot design, where the two parental inbreds were included as controls. The entire experiment was replicated b = 3 times.

For each IRIL, shoot samples of four plants from one pot were pooled so that each IRIL was represented by one sample for each of the three replicates in each of the two Fe regimes. Afterwards, the samples were ground and Al, B, Cd, Ca, Cu, Fe, Mg, Mn, Mo, Ni, P, K, Na, S, and Zn concentrations were measured using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 6000 SERIES, Thermo Fisher) according to Shi et al. (2012). Additionally, morphological and physiological traits evaluated in a companion study (Benke et al., 2014) were included into the current analysis.

Statistical analyses

Data on mineral element concentrations under each Fe regime were analyzed using the following mixed model:

$$y_{ik} = \mu + g_i + r_k + e_{ik} \quad ,$$

where y_{ik} was the mean of the ith genotype in one pot of the kth replication, μ the general mean, g_i the effect of the ith genotype, r_k the effect of the kth replication, and e_{ik} the residual error.

Table 2 Morphological and physiological traits predicted by linear combinations of mineral nutrients for the deficient and sufficient iron regimes. $\%r^2$ is the proportion of the phenotypic variance explained by the selected variables.

	Deficient		Sufficient	
Traits	Optimum linear model	$\%r^2$	Optimum linear model	$\%r^2$
SP3	38.244 − 13.122Cd − 1.312P	0.382	33.911 + 0.081Fe − 1.097P	0.145
SP4	28.943 − 18.670Cd + 0.135Fe − 1.572P	0.488	38.479 + 0.070Fe − 2.126P + 0.111Zn	0.344
SP5	36.979 − 0.479Al − 0.474B + 0.257Fe − 0.080Mn − 0.219K	0.574	45.461 − 2.459P + 0.168Zn	0.263
SP6	34.377 − 0.347B + 0.198Fe − 0.061Mn − 0.775P − 0.183K	0.568	31.308 − 1.851P + 5.892S	0.251
RL	76.879 + 1.806Ca − 1.612P − 6.779S	0.253	72.543 − 0.141Mn	0.041
RW	11.448 − 0.131Al + 0.617Mg − 0.372P − 0.056K	0.499	9.367 + 0.256Ca − 1.054P + 0.045Zn	0.390
SL	26.671 − 9.839Cd + 0.094Fe − 0.817P	0.376	26.993 + 1.600Mg − 1.045P	0.173
SDW	3.176 − 0.027Al − 0.005Mn − 0.06P − 0.012K	0.599	2.324 − 0.230P + 0.475S	0.399
H_2O	92.785 − 0.017Fe + 0.027K	0.158	94.111 − 0.143B + 0.167Mo	0.153
SDW/SL	0.086 − 0.001Mn − 0.002P	0.484	0.083 + 0.319Co − 0.008Mg − 0.008P + 0.023S	0.313
BTR	8.380 − 0.142B + 0.799Mg − 0.319P	0.316	−	−
LAT	7.134 − 2.740Cd + 0.157Mo − 0.291P	0.433	9.552 − 4.874Cd + 0.032Mn − 1.041P + 0.033Zn	0.535
NEC	9.454 − 0.080Al + 0.009Mn − 0.178P	0.193	8.750 − 0.178P	0.049

To estimate adjusted entry means (AEM) for all genotypes, g_i was considered as fixed and r_k as random at each of two Fe regimes. Furthermore, g_i and r_k were considered as random to estimate the genotypic variance (σ_g^2), the error variance (σ_e^2), and perform a significance test of the genotypic variance. All mixed model calculations were performed with ASReml (Gilmour et al., 2006).

The broad sense heritability H^2 for each Fe regime was calculated as:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{b}} \ .$$

The AEM of all genotypes for all mineral elements at both Fe regimes were tested with a Kolmogorov-Smirnov test (Chakravarti et al., 1967) for their normal distribution. Pairwise correlation coefficients were assessed between all pairs of mineral elements examined in the current study as well as morphological and physiological traits evaluated by Benke et al. (2014). Student's t-test was calculated between deficient and sufficient Fe regimes. Spearman rank correlation was used to correlate the mineral elements concentration measured for IRILs under deficient Fe regime with the mineral elements concentration under sufficient Fe regime.

Multiple stepwise regression was performed for each morphological and physiological trait at deficient and sufficient Fe regime as dependent variable and mineral elements of the same Fe regime were used as independent variables. Variable selection was performed based on the Bayesian information criterion (BIC) (Schwarz, 1978) for the following model:

$$y_i = \mu + \sum_{p=1}^{v} b_p x_{pi} + e_i \ ,$$

where y_i was the observation of the ith genotype, μ the intercept term, v the number of selected variables, b_p the regression coefficient of the pth mineral element, x_{pi} the AEM of the pth mineral element for the ith genotype, and e_i the residual.

Genetic map

The publicly available genotypic data (http://www.maizegdb.org/map.php) for the IRILs were used in our study. The genetic map positions of these markers on the IBM2 map are publicly available (http://www.maizegdb.org/map.php) and were the basis of our analyses. A total of 336 markers were excluded that showed a highly significant ($P<0.001$) distorted segregation (*cf.* Dufour et al., 2001). The remaining 1652 markers were used for the QTL analyses. Missing genotypic information in our marker set was imputed as described by Sen and Churchill (2001).

QTL analyses

Due to the high number of available markers, cofactors could not be selected using standard stepwise regression.

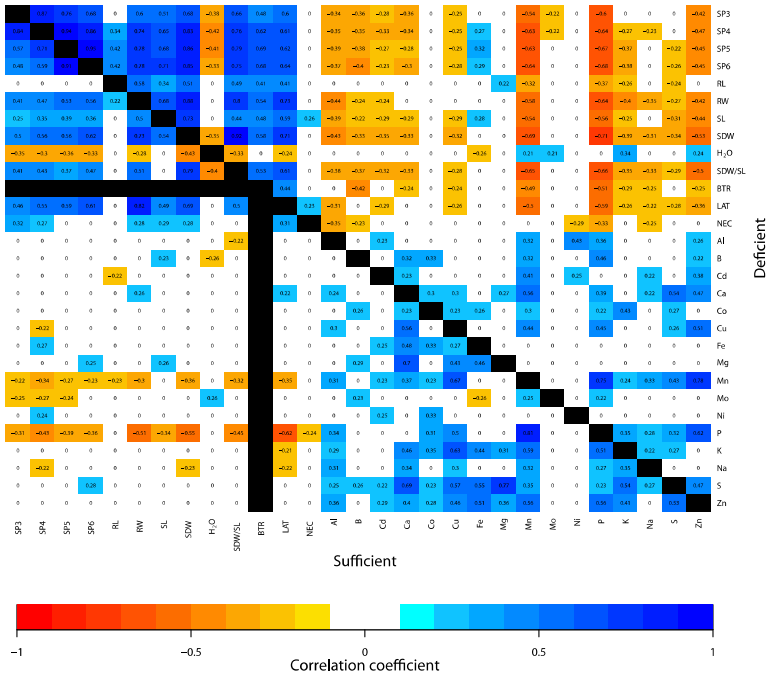

Fig. 2 Pairwise correlation coefficients calculated between all pairs of mineral nutrients concentrations and morphological and physiological traits using the maize IBM population. The significant (α = 0.05) values above the diagonal represent the correlation coefficients between the adjusted entry means (AEM) of the deficient iron regime. The significant (α = 0.05) values below the diagonal represent the correlation coefficients between the AEM of the sufficient iron regime.

Therefore, the following procedure was applied for each trait. One random marker was selected from each bin. Multiple stepwise regression was used to select cofactors from this set of markers based on the Bayesian information criterion (BIC) (Schwarz, 1978). This procedure was repeated 1000 times. The average number of selected cofactors across the 1000 times repetition was used as estimator for the number of bins to study in more detail. Out of these bins, 100 markers were chosen randomly and the final set of cofactors based on BIC was selected.

A total of 1000 permutation runs were performed for each trait and Fe regime to determine the α = 0.05 experiment-wise type I error for a QTL (Churchill and Doerge, 1994). The 95% Bayesian confidence interval was calculated for each QTL location (Sen, 1998). The confidence interval was expanded to the nearest flanking markers and their physical map localization was derived from B73 RefGen_v2_sequence to be able to extract all putative genes from a defined interval.

If not stated differently, all analyses were performed using statistical software R (R Core Team, 2012).

RESULTS

For all mineral elements examined in our study, a significant (α = 0.05) genotypic variance was observed for both Fe regimes except for Ni under the deficient Fe regime. The broad sense heritabilities for the mineral element concentrations evaluated under the deficient Fe regime ranged from 0.30 (Ni) to 0.95 (Mo) (Table 1). For the

Table 3 Summary of the quantitative trait loci (QTL) detected using the maize IBM population evaluated under a deficient iron regime, where Chr. is the chromosome, Pos. the position in centi Morgan on the genetic map, Add. the additive effect, $\%r^2$ the percentage of the explained phenotypic variance, and genetic map interval of the flanking markers with corresponding physical map interval including potential genes according to the filtered gene set B73 RefGen_v2 for the corresponding QTL confidence interval.

Trait	QTL	Chr.	Pos. (cM)	Add.	$\%r^2$	Genetic map interval	Flanking markers	Physical map interval	Genes
Cd	1	2	374.0	-0.11	49.66	373.5 - 375.3	bnlg1036 - umc1658	163,316,218 - 163,566,033	3
				Total	49.66				
Ca	1	1	200.0	-1.39	11.33	199.7 - 201.5	ufg78 - cdo1387b	27,398,858 - 27,918,358	11
Ca	2	4	476.0	1.11	6.68	475.7 - 476.0	dupssr28 - mmp70	184,802,865 - 185,569,074	27
Ca	3	4	738.0	1.18	0.43	737.8 - 739.3	bip2 - umc1649	239,833,964 - 240,170,285	16
Ca	4	4	746.0	-2.33	1.57	744.1 - 748.3	cat3 - umc1707	240,105,137 - 240,619,080	25
				Total	24.82				
Co	1	3	728.0	-0.01	14.31	699.2 - 728.1	a1 - jpsb107c	216,304,734 - 219,248,887	98
Co	2	6	387.3	0.01	5.19	387.1 - 393.0	expa5 - IDP7503	153,502,318 - 153,555,627	0
Co	3	8	316.0	0.01	15.94	312.4 - 320.6	umc1858 - AY104017	111,185,873 - 119,043,415	146
				Total	28.59				
Cu	1	5	372.0	2.62	8.32	371.2 - 376.4	csu308 - incw1	168,646,501 - 169,459,090	15
Cu	2	5	398.0	1.04	1.32	397.0 - 402.2	gst17 - mmp47	174,134,238 - 175,787,174	69
Cu	3	7	310.0	2.22	6.78	309.9 - 310.6	bnl15.21 - bnl5.46c	131,984,321 - 132,539,734	15
Cu	4	7	364.0	-3.51	15.34	361.9 - 364.8	umc56 - uaz221	143,169,908 - 143,389,581	8
Cu	5	9	16.0	2.64	10.16	14.0 - 17.7	npi253a - umc1370	4,301,006 - 4,434,692	6
				Total	45.09				
Fe	1	1	816.0	6.08	7.53	815.2 - 821.5	ufg53 - csu696	252,209,343 - 253,570,780	29
Fe	2	1	836.0	2.37	1.11	833 - 839.3	chrom7 - glb1	256,342,909 - 258,365,809	56
Fe	3	3	8.0	2.72	2.51	7.5 - 11.0	phi453121a - phi404206	1,591,607 - 2,084,934	27
Fe	4	9	316.0	-3.76	5.37	315.7 - 317.0	umc2121 - umc38c	124,251,796 - 127,743,532	90
				Total	26.72				
Mg	1	5	334.0	0.24	5.74	332.7 - 336.5	mmp19 - AY110906	158,820,967 - 162,668,584	64
Mg	2	9	298.0	-0.17	2.05	290.1 - 298.0	gta101c - expb8	113,013,688 - 119,479,379	133
Mg	3	9	326.0	-0.20	2.74	322.6 - 326.0	umc1078 - ufg13a	130,485,871 - 133,595,334	62
				Total	18.43				
Mo	1	1	802.0	-2.65	55.85	800.7 - 805.3	umc1991 - umc1843	245,259,400 - 248,825,948	86
Mo	2	2	534.0	-0.39	1.28	529.8 - 536.5	bnl6.20 - npi61	212,348,455 - 213,677,940	56
Mo	3	7	148.0	-0.77	5.06	132.0 - 148.5	asg34a - gta101a	13,954,636 - 14,698,304	13
				Total	72.79				
P	1	1	824.0	-0.05	0.01	821.5 - 825.8	csu696 - rz403	253,570,111 - 256,342,908	57
P	2	1	848.0	-1.64	5.20	847.3 - 864.6	csu222a - umc197a	257,875,680 - 262,946,924	148
P	3	1	1120.0	-0.86	3.62	1119.2 - 1121.9	umc1819 - tufm1	298,563,294 - 300,485,133	58
P	4	2	348.0	1.03	4.66	339.3 - 349.0	umc1454 - asrp2	69,703,905 - 140,919,225	669
P	5	5	210.0	1.10	4.49	204.1 - 210.3	rz474a - umc1686	16,131,458 - 17,817,322	35
P	6	5	260.0	-1.46	8.25	257.8 - 260.2	umc2295 - umc1315	38,173,734 - 42,303,953	64
P	7	7	174.0	-2.43	23.00	170.8 - 178.0	crt2 - AY110473	24,410,021 - 50,154,299	309
				Total	61.76				
K	1	7	158.0	0.60	0.04	156.9 - 158	umc1978 - umc2327	20,778,431 - 21,077,079	3
K	2	7	170.0	-4.94	2.52	168.5 - 170.8	umc1927 - crt2	16,889,863 - 24,413,947	132
				Total	9.62				
Na	1	1	166.0	-15.07	5.92	165.8 - 166.0	umc2226 - mmp135	21,419,789 - 22,003,995	20
Na	2	1	892.0	-22.54	11.31	890.9 - 902.1	AY110019 - IDP9012	270,964,968 - 274,280,008	74
Na	3	4	452.0	21.81	11.06	449.4 - 452.1	umc1667 - AY109534	179,742,246 - 181,230,035	39
				Total	24.36				
S	1	2	116.0	0.25	6.12	94.4 - 122.4	AY109516 - agrc539a	8,897,793 - 10,545,409	71
S	2	3	324.0	0.18	4.46	319.8 - 325.4	pza00920 - IDP474	143,028,421 - 148,223,783	103
S	3	5	662.0	0.29	11.56	661.8 - 664.3	rz446b - gpm745b	205,440,973 - 217,012,402	516
S	4	7	180.0	-0.25	8.24	179.9 - 180.5	cyp6 - bnlg1094	17,101,442 - 50,173,540	439
S	5	10	202.0	0.26	9.09	200.5 - 203.0	AY110248 - ufg59	66,720,368 - 76,238,103	134
				Total	49.47				
Zn	1	4	270.0	-12.65	0.40	268.4 - 270.3	bnlg1265 - umc1382	39,318,672 - 45,069,173	97
Zn	2	4	278.0	-24.56	1.53	277.8 - 279.9	psr152b - nnr1	46,450,572 - 56,313,225	92
Zn	3	4	492.0	34.27	7.90	487.7 - 499.9	AY112127 - ufg23	185,731,904 - 186,679,900	30
Zn	4	7	164.0	-41.41	12.67	162.4 - 167.4	AY105589 - tug9	17,029,068 - 21,464,802	82
				Total	32.42				

sufficient Fe regime, the same trend was observed and the broad sense heritabilities varied between 0.35 (Ni) and 0.95 (Mo).

The AEM for all mineral elements except Fe and Na was on average across all IRILs significantly higher (α = 0.05) under the deficient Fe regime compared to the sufficient regime (Fig. 1). In contrast, the mineral element concentration of Fe was significantly (α = 0.05) lower for the deficient Fe regime than for the sufficient. For Na, no significant (α = 0.05) difference was observed between the two Fe regimes on average across all IRILs. The parental inbred Mo17 showed under the deficient Fe regime for B, Cd, Mg, Mn, Mo, Ni, P, Na, and Zn a higher AEM than the parental inbred B73. For Al, Ca, Co, Cu, Fe, K, and S the opposite was true. Furthermore, a higher mineral element accumulation under the sufficient Fe regime was observed for Mo17 in comparison to B73 with the exception of Co, K, Na, and Zn. Spearman's rank correlation coefficient (ρ) between mineral element concentrations observed under both Fe regimes ranged from -0.04 (Co) to 0.89 (Mo). The pairwise correlation coefficients calculated for the morphological and physiological traits and mineral element concentrations measured in the deficient Fe regime ranged from -0.71 (between SDW and P) to 0.78 (between Mn and Zn) (Fig. 2). By comparison, for the sufficient Fe regime, the strongest negative correlation was observed between LAT and P (-0.62) and the highest between Mn and P (0.81) (Fig. 2).

The percentage of the phenotypic variance (%r^2) of the morphological and physiological traits which could be explained by variation of a selected set of mineral elements ranged for the deficient Fe regime from 0.158 (H_2O) to 0.599 (SDW), whereas 11 mineral elements (Al, B, Cd, Ca, Fe, Mg, Mn, Mo, P, K, and S) were related to the morphological and physiological trait formation (Table 2). For the sufficient Fe regime, the %r^2 ranged from 0.041 (RL) to 0.535 (LAT) and another set of 11 mineral elements (B, Cd, Ca, Co, Fe, Mg, Mn, Mo, P, S, and Zn) was related to the statistical phenotypic prediction.

The QTL analyses of the concentration of mineral elements measured under the deficient Fe regime revealed a total of 44 QTLs (Table 3). The highest number of QTLs for the deficient Fe regime was detected for P (7) while it was zero for Al, B, Mn, and Ni. The phenotypic variance explained by the QTL was highest for Mo QTL1 (55.9%). The maximum percentage of phenotypic variance explained in a simultaneous fit by all QTLs for one mineral element was 72.8% (Mo) and the minimum was 9.6% (K). The allele increasing the mineral element concentration was contributed by Mo17 at 23 of the 44 QTLs.

Under the sufficient Fe regime, a lower number of QTLs (13) was detected across all mineral elements compared to the deficient Fe regime (Table 4). The number of QTLs ranged from 5 (Mo) to 0 (B, Ca, Co, Mg, Mn, Ni, K, Na, and Zn). The percentage of phenotypic variance explained by the QTL showed for QTL1 of Mo the highest value (59.1%). The proportion of phenotypic variance explained in a simultaneous fit by all QTLs for one mineral element was at maximum for Mo (75.49%) and at minimum for Al (10.4%). The additive effect of the QTLs indicated for 10 of the 13 QTLs that the trait increasing allele was contributed by Mo17.

The size of the QTL confidence intervals ranged from 28.9 cM for QTL1 of Co to 0.2 cM for QTL1 of Na under the deficient Fe regime (Table 3). The largest QTL confidence interval was detected for QTL4 of Mo (43.1 cM) and the smallest for QTL3 of P (0.7 cM) under the sufficient Fe regime. The physical size of the confidence interval of P QTL4 included 71,215,321 bp corresponding to 669 genes for the deficient Fe regime. The confidence interval of Mo QTL4 showed within 79,219,531 bp 742 genes for the sufficient Fe regime.

The QTL confidence intervals detected for concentrations of multiple mineral elements clustered in regions on chromosome 1 and 7 (Fig. 3). The cluster on chromosome 1 comprised 10 QTLs from both Fe regimes and was located between position 800 and 900 cM that is corresponding to physical map interval from 245,259,400 to 274,280,008.

The *NAS3* and *ZIP10* genes mapped to the confidence interval of QTL2 for P under the deficient Fe regime (Fig. 3). The gene *HMA3* was located within the Cd QTL confidence interval under both Fe regimes. Furthermore, the confidence interval of S QTL2 detected under the deficient Fe regime comprised the genes *SBAS* and *IRO2*. The *KCH2* gene mapped within the QTL2 confidence interval of P identified in the sufficient Fe regime.

DISCUSSION

To determine differences in mineral element profile in maize leaves we used the natural variation to genetically dissect this trait under deficient or sufficient Fe supply. Furthermore, we examined whether it is possible to relate morphological traits from mineral element concentrations. As interactions with Fe have been reported for several mineral elements (Kanai et al., 2009; Zheng et al., 2009; Mori and Nishizawa, 1987; Astolfi et al., 2003), the rationale behind our approach was to identify those loci which make a major contribution to a balanced element homeostasis when plants are subjected to Fe deficiency.

Natural variation in the mineral element profile of maize leaves

We observed that the mineral element concentrations in maize leaves under both Fe regimes were significantly influenced (α = 0.05, except Ni under the 10 μM regime) by genetic variation. Hence, we determined the proportion of the phenotypic variation which was explained by genotypic variance (H^2, Table 1). We observed a 20% lower H^2 for the elements B, P, S, and Zn in the sufficient Fe regime compared to the deficient Fe regime. This observation was caused by a larger genotypic variation under the Fe-deficient regime for B, P, S, and Zn compared to the Fe-sufficient regime, which was most likely due to a more tightly regulated nutrient acquisition under Fe sufficient growth conditions. Furthermore, this finding suggests that our study will have a higher statistical power for QTL detection in Fe-deficient than in Fe-sufficient plants.

The concentration of all mineral elements, except Na, was significantly (α = 0.05) affected by the Fe regime (Fig. 1). We observed for all mineral elements except Fe a higher accumulation in the leaves of Fe-deficient

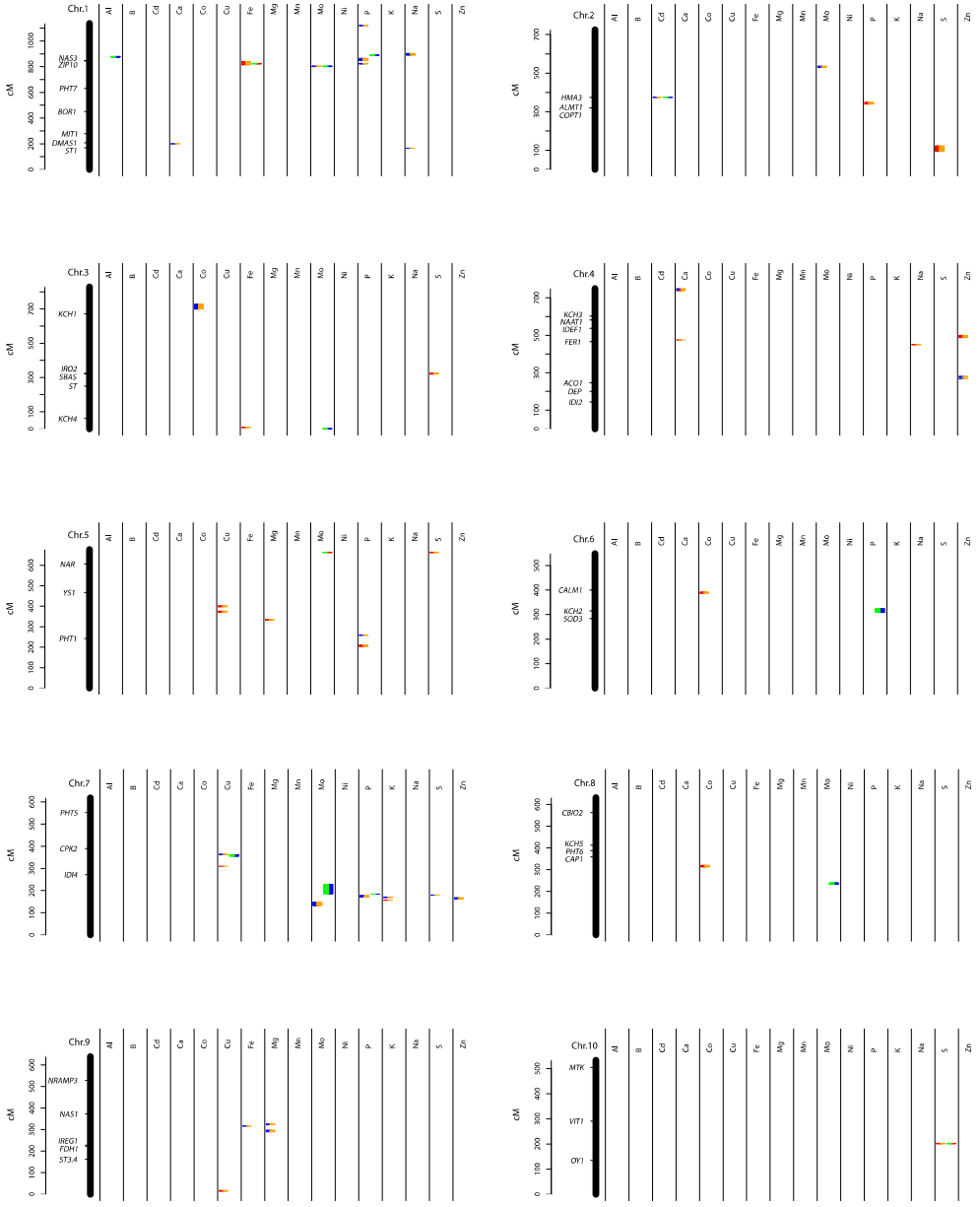

Fig. 3 Projection of 43 genes suggested being responsible for ion homeostasis on the IBM2 map. The confidence intervals of the quantitative trait loci detected for the concentrations of 16 mineral nutrients of our study for deficient and sufficient iron regimes are represented by orange and green bars, respectively. The trait value increasing alleles are indicated as blue and red bars for Mo17 and B73, respectively.

than of Fe-sufficient plants. Our finding is in accordance with results of Thoiron et al. (1997) who observed in Fe-deprived maize plants a lower Fe concentration in leaves, whereas Cu, Mn, Mg, and Ca concentrations increased. However, the concentration of K showed no alteration in the study of Thoiron et al. (1997), whereas for the IRILs in the current study an accumulation of K was observed under the Fe deficiency regime. This discrepancy might be due to the different Fe regimes as well as the different maize germplasm used in these two studies. The accumulation of macroelements like K, Ca, and Mg most likely arose from a concentration effect due to suppressed leaf biomass formation under Fe deficiency (Marschner, 2012). This situation is different for some of the metal cations. The study of Kanai et al. (2009) showed a 15-fold higher accumulation of Zn in maize leaves harvested under Fe-deficient than those obtained under Fe-sufficient conditions. Furthermore, Meda et al. (2007) showed that Cd accumulated in Fe-deficient maize leaves to a much larger extent than under sufficient Fe supply. The accumulation of these and other divalent metal cations is a well-established phenomenon which is most likely caused by a lacking specificity of the Fe transport systems in the root plasma membrane, irrespective of whether ferrous Fe is taken up by IRT-related Fe^2 transporters or chelated Fe is taken up by metal-phytosiderophore transporters, since both types of transporters show a low specificity for the transported metal or metal-complex (Schaaf et al., 2004; Rogers et al., 2000)

We observed under Fe deficiency an approximately 25-50% higher accumulation of S and P than under the Fe sufficient regime (Fig. 1). The accumulation of S was in accordance with results of Zuchi et al. (2012) who observed in wheat an increased phytosiderophore exudation at higher S supply probably due to a higher Fe demand and more efficient nicotianamine biosynthesis which depends on S-adenosyl-methionine and the S-dependent Yang cycle. In contrast, P undergoes an antagonistic interaction with Fe, in which an increasing P supply decreases Fe uptake and Fe availability and promotes Fe deficiency symptoms (Zheng et al., 2009).

To examine if the maize IRILs accumulated mineral elements under both Fe regimes by overlapping mechanisms, we performed a rank correlation test (Fig. 1). We observed for all mineral elements, except for Co, K, and Ni, moderately to highly significant ($\alpha = 0.05$; $\alpha = 0.001$) rank correlations between both Fe regimes. This suggested a linked inheritance and/or pleiotropic genetic control of mineral element uptake and accumulation mechanisms in maize leaves. In contrast, the absence of significant mineral element rank correlations for Co, K, and Ni suggested a different genetic control of their homeostasis under the two Fe regimes.

Mineral elements affecting the expression of phenotypic traits

The elevation of the concentration of several mineral elements under Fe deficiency might also affect the expression of morphological and other physiological traits (Baxter et al., 2008). We determined the optimum linear model to predict morphological and physiological traits from linear combinations of mineral elements (Table 2). We observed that each morphological and physiological trait showed an individual mineral element constellation that explained to a considerable proportion the phenotypic variance. This suggested that the change in the concentrations of certain mineral elements modulated morphological and physiological traits. Furthermore, our observation suggested that in case when morphological and physiological traits are difficult to measure due to the inaccessibility of this organ (i.e. root), its status could be predicted based on the associated mineral element signature (Baxter et al., 2008).

Under Fe deficiency Fe supply negatively affected the phenotypic trait formation of H_2O (Table 2). This finding was supported by the pairwise negative correlation between Fe and H_2O (Fig. 2). This finding might be explained by an Fe deficiency-induced decrease in transpiration. In peach, Fe deficiency has been reported to cause a lower leaf xylem vessel size leading to lower xylem conductivity (Eichert et al., 2010). The results of the multiple regression analysis as well as the pairwise correlation analysis suggested that an increased Fe concentration is associated with increased trait values for the morphological and physiological traits SP4, SP5, SP6, and shoot length under Fe-deficient growth (Table 2, Fig. 2). The chloroplast with its large demand for Fe in photosynthetic electron transport and its large storge capacity for Fe in the form of ferritin creates the most important sink for Fe in plants (Briat et al., 1999). This indicates that under Fe deficiency available Fe might be assimilated completely in the leaves 4, 5, and 6. Consequently, the determination of mechanisms responsible for a more efficient Fe utilization become more emphasis. Moreover, the determination of genes responsible for mineral element homeostasis at either Fe regime provides an additional insight into the physiology of Fe homeostasis in maize. As genetic markers for relevant loci for mineral nutrient homeostasis are of enormous interest for marker-assisted selection, the present analysis may provide a first step to reach this goal via QTL mapping.

Mineral homeostasis dissected by QTL mapping

We observed that in the analysis of Baxter et al. (2014) similar QTLs were detected in maize kernels for Cd, Mo, P, and Zn.This observation indicates that the mechanisms contributing to the sequestration and accumulation of these mineral elements in maize leaves (Table 3, Table 4) operate along the similar lines as in maize kernels.

For the accumulation of Cd in maize leaves, we detected under both Fe regimes the same QTL explaining about 50% of the phenotypic variance (%r2). Under both Fe regimes the trait-increasing allele was provided by the Fe-inefficient parental inbred Mo17 that accumulated 5 times more Cd in comparison to the Fe-efficient parental inbred B73 (Table 3, Table 4, Fig. 1). This most likely reflects the more severe Fe deficiency experienced by Mo17 relative to B73 (*cf.* Benke et al., 2014). We observed that the putative vacuolar Cd transporter (GRMZM2G455491) *HMA3* (Gravot et al., 2004; Miyadate et al., 2011) was included in the Cd QTL confidence interval on chromosome 2 (Fig. 3). Miyadate et al. (2011) observed that

in rice *OsHMA3* mediates vacuolar Cd sequestration in roots which decreases the Cd translocation to the leaves. In analogy, our results suggest that the same may hold true for *ZmHMA3*. A more efficient sequestration of Cd in roots of B73 relative to Mo17 might may reduce the competition between Cd and Fe in leaves and thereby suppress Fe deficiency symptoms.

Table 4 Summary of the quantitative trait loci (QTL) detected using the maize IBM population evaluated under a sufficient iron regime, where Chr. is the chromosome, Pos. the position in centi Morgan on the genetic map, Add. the additive effect, %r^2 the percentage of the explained phenotypic variance, and genetic map interval of the flanking markers with corresponding physical map interval including potential genes according to the filtered gene set B73 RefGen_v2 for the corresponding QTL confidence interval.

Trait	QTL	Chr.	Pos. (cM)	Add.	%r^2	Genetic map interval	Flanking markers	Physical map interval	Genes
Al	1	1	878.0	-2.07	10.44	874.3 - 879.7	csu554a - mmp195d	266,933,205 - 267,210,124	9
				Total	10.44				
Cd	1	2	374.0	-0.11	49.66	373.5 - 375.3	bnlg1036 - umc1658	163,316,218 - 163,566,033	3
				Total	49.66				
Cu	1	7	356.0	-3.06	14.71	354.9 - 361.9	npi389 - umc56	143,169,908 - 143,389,581	8
				Total	14.71				
Fe	1	1	824.0	6.68	15.55	821.5 - 825.8	csu696 - rz403	253,570,111 - 256,342,908	57
				Total	15.55				
Mo	1	1	802.0	-2.73	59.11	800.7 - 805.3	umc1991 - umc1843	245,259,400 - 248,825,948	86
Mo	2	3	2.0	-0.41	1.44	0.0 - 2.0	umc2118 - g2	1,200,876 - 1,469,113	13
Mo	3	5	664.0	0.18	0.28	661.8 - 664.3	rz446b - gpm745b	205,440,973 - 217,012,402	516
Mo	4	7	200.0	-1.37	6.66	185.6 - 228.7	rz698d - AY109968	21,437,175 - 100,656,705	742
Mo	5	8	234.0	-0.34	0.96	232.9 - 240.7	umc2355 - pco098406	76,558,017 - 95,150,703	249
				Total	75.49				
P	1	1	890.0	-1.81	13.99	887.5 - 890.9	cdo122a - AY110019	263,079,095 - 270,965,223	194
P	2	6	323.5	-1.17	6.84	308.6 - 325.4	pge20 - mbd120	141,251,542 - 147,345,458	143
P	3	7	184.0	-2.39	27.22	183.7 - 184.4	npi111 - mmp26	50,078,806 - 50,149,169	3
				Total	41.47				
S	1	10	202.0	0.37	19.29	200.5 - 203.0	AY110248 - ufg59	66,720,368 - 76,238,103	134
				Total	19.29				

Under Fe deficiency, we detected in the interval of Fe concentration QTL2 on chromosome 1 the gene *ZIP10* (GRMZM2G118821) which encodes a putative Zn transporter. Talke et al. (2006) showed that in *Arabidopsis thaliana ZIP10* is responsible for Zn uptake into root cells. Furthermore, Urbany et al. (2013) showed in a RNA-Sequencing approach for B73 and Mo17 under Fe-deficient and Fe-sufficient conditions that *ZIP10* was 2-fold higher expressed in B73 than in Mo17. This observation may indicate that *ZIP10* is of importance for Zn uptake in Fe-deficient maize. On the other hand, ZIP10 may also contribute to Fe^{2+} uptake as ZIP10 is 87% homologous to the rice Fe^{2+} transporter *OsIRT1*. However, this requires further functional studies. We further found under Fe deficiency on chromosome 3 within the confidence interval of Fe QTL3 a glutaredoxin (GRMZM2G449160) gene (Table 3, Fig. 3). Herrero and De La Torre-Ruiz (2007) suggested for two glutaredoxin genes (Grx3 and Grx4) a regulative contribution to cellular Fe homeostasis and protection against oxidative stress in yeast. The glutaredoxin gene might play a particular role in redox homeostasis in Fe-deficient maize. Again, biochemical validation analyses are necessary to confirm such a functional link.

We further detected under Fe sufficiency one QTL for leaf Fe concentration that includes a putative *NRAMP2* (GRMZM2G025680) gene of maize (Table 4, Fig. 1). Curie et al. (2000) observed that the transcript of its Arabidopsis homologue is highly expressed during Fe sufficiency in roots. In contrast, in rice *NRAMP2* expression was observed exclusively in leaves. Due to the fact that *NRAMP2* has a broad substrate specificity that includes Cu, Mn, Ni, and Zn besides Fe (Hall and Guerinot, 2006) it might also contribute to the general homeostasis of microelements in shoot cells of plants growing under a sufficient Fe supply.

Besides Fe homeostasis, the balance of other essential mineral nutrients is indispensable for the plant to avoid repressive interactions. We observed the highest %r^2 for the QTL7 of the mineral element concentration of P under the Fe deficiency regime (Fig. 3, Table 3). We detected within the confidence interval of this QTL7 the phosphate transporter homologue *PHT1;5* (GRMZM2G139639). Nagarajan et al. (2011) observed that in Arabidopsis PHT1;5 contributes to the translocation of P from source leaves to sink organs and in particular to roots of plants adequately supplied with P. Supposed that the homolog in maize plays a similar role, PHT1;5 might contribute to a depletion of P from those sites or compartments where the negative interaction with P impairs the Fe nutritional status in maize leaves. Such a contribution of *PHT1;5* was not indicated under the Fe-deficient regime.

On chromosome 5, in the confidence interval of the Mg QTL detected under the deficient Fe supply, a Mg-dependent protein phosphatase 2C homolog (PP2C) (GRMZM2G102560) was identified (Fig.3, Table 3). Xue et al. (2008) reported that members of the PP2C group are associated with a negative modulation of protein kinase pathways activated particularly under certain environmental stresses. Therefore, the detected maize gene might be

interesting for biochemical analyses in order to reveal its involvement in the mineral element homeostasis under Fe deficiency.

In the QTL2 interval of Zn concentration under the Fe-deficient regime, we detected the gene *ZIP4* (GRMZM2G045531) (Fig. 3, Table 3). A homolog to this gene was identified by Grotz et al. (1998) in *Arabidopsis thaliana* where it was predicted to play a role in plastidic Zn transport. Furthermore, the expression of *ZIP4* was downregulated under Fe-deficient growth conditions in Arabidposis (Wintz et al., 2003). Interestingly, in the RNA-sequencing analysis by Urbany et al. (2013) an even stronger downregulation under Fe deficiency was observed for Mo17 than for B73 for an unknown gene (GRMZM2G425999) included in the Zn QTL2 interval. This might lead to an accumulation of Zn in the cell due to a putative negative regulation of *ZIP4* by Fe (Wintz et al., 2003). Furthermore, excess Zn accumulation leads to more severe Fe deficiency (Wintz et al., 2003; Kanai et al., 2009). Therefore, the exploration of genes responsible for Zn/Fe homeostasis would present an important step towards improving the nutrient status in Fe-deficient maize.

Conclusions

In this study we provide a detailed analyses of the mineral element homeostasis in maize plants under two different Fe regimes. The deficient Fe regime caused a significant accumulation of essential and harmful mineral elements in maize leaves that was presumably caused by a concentration effect or by the low substrate specificity of the Fe uptake system in maize roots. All mineral elements, except for Co, K, and Ni, showed a pleiotropic genetic control under both Fe regimes. By QTL mapping we identified loci that contribute to the variation in mineral element homeostasis, including genes that sequester Cd in vacuoles (*HMA3*), transport Zn^{2+} into root cells (*ZIP10*), protect cells against oxidative stress (glutaredoxin), ensure micronutrient homeostasis at ample Fe supply (*NRAMP2*), or prevent maize leaves from excess accumulation of antagonistically acting elements like P or Zn (*PHT1;5*, *ZIP4*).

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Table S1 Genes, which were projected on the IBM2 genetic map of maize (Fig. 3).

Ion	Gene name	Gene	NCBI accession	Bin	Gene function	Reference
Al	Aluminum-induced transporter 1	*ALMT1*	NM_001112522.1	2.04	Mediate aluminum-activated organic acid responses	Piñeros et al. (2008)
B	Boron transporter 1	*BOR1*	EU972085.1	1.05	Boron transport	Nakagawa et al. (2007)
Cd	Heavy metal transporter 3	*HMA3*	NM_001196320.1[a]	2.06	Root-to-shoot cadmium translocation	Miyadate et al. (2011)
Ca	Calmodulin 1	*CALM1*	NM_001111985.1	6.06	Calcium/calmodulin-mediated signal network	Griess et al. (1994)
	Calcium dependent protein kinase 2	*CPK2*	NM_001112072.1	7.03	Protein phosphorylation	Patil et al. (1995)
	Calcium pump 1	*CAP1*	NM_001111452.1	8.05	Ca transport stimulated by calmodulin	Subbaiah and Sachs (2000)
Co	Cobalt import ATP-binding protein 2	*CBIO2*	EU973936.1	8.08	Cobalt import	Alexandrov et al. (2009)
Cu	Copper transporter 1	*COPT1*	EU952941.1	2.04	Copper transport	Yuan et al. (2011)
Fe	Aconitase 1	*ACO1*	NM_001143012.1[a]	4.04	Catalyzes citrate to isocitrate	Beinert et al. (1996)
	Dehydratase-enolase-phosphatase	*DEP*	BT042841.1[a]	4.03	Involved in methionine (Met) recycling process	Kobayashi et al. (2005)
	2′-deoxymugineic acid synthase 1	*DMAS1*	AB269909.1	1.03	Reduces the 3″-oxo acid form to deoxymugineic acid	Bashir et al. (2006)
	Formate dehydrogenase 1	*FDH1*	EU967680.1	9.03	Reduces nicotinamide adenine dinucleotide (NAD^+) to NADH	Suzuki et al. (1998)
	Ferritin 1	*FER1*	NM_001112093.1	4.08	Iron storage protein	Savino et al. (1997)
	Iron deficiency-responsive cis-acting element binding factor 1	*IDEF1*	BT038952.1[a]	4.08	Gene regulation during Fe-deficiency	Kobayashi et al. (2009)
	Methylthioribose-1-phosphate isomerase	*IDI2*	EU970346.1	4.03	Involved in Met recycling process	Yamaguchi et al. (2000)
	Asparate/tyrosine/aromatic aminotransferase	*IDI4*	EU945835.1[a]	7.02	Involved in Met recycling process	Suzuki et al. (2006)
	Iron-regulated bHLH transcription factor 2	*IRO2*	DQ245918.1[a]	3.05	Gene regulation	Ogo et al. (2011)
	Mitochondrial iron transporter 1	*MIT1*	BT042306.1[a]	1.03	Tranpsorts iron	Bashir et al. (2011)
	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	*MTN*	BT065125.1[a]	1.06	Hydrolyses the glycosidic bond between ribose and adenine	Rzewuski et al. (2007)
	Methylthioribose kinase	*MTK*	EU963151.1	10.07	Involved in Met recycling process	Sauter et al. (2004)
	Nicotianamine aminotransferase 1	*NAAT1*	AB375372.1	4.09	Catalyzes amino transfer on nicotianamine	Inoue et al. (2008)
	Nicotianamine synthase 1	*NAS1*	AB061270.1	9.05	Catalyzes conjugation of three S-adenosylmethionine molecules	Mizuno et al. (2003)
	Nicotianamine synthase 3	*NAS3*	AB042551.1	1.09	Catalyzes conjugation of three S-adenosylmethionine molecules	Mizuno et al. (2003)
	Natural resistance associated macrophage protein 3	*NRAMP3*	EU966388.1	9.06	Vacuolar Fe mobilization in the seedling development	Lanquar et al. (2005)
	High affinity sulfate transporter 1	*ST1*	AF355602.1	1.02	Transports sulfate from the root apoplast	Hopkins et al. (2004)
	Vacuolar iron transporter 1	*VIT1*	EU968723.1[a]	10.04	Transports iron into the vacuole of provascular cells	Kim et al. (2006)
	Yellow stripe 1	*YS1*	AF186234.2	5.05	Iron-phytosiderophore-complex transporter	Curie et al. (2001)
Mg	Oil yellow 1	*OY1*	DQ084025.1	10.02	Magnesium chelation	Sawers et al. (2006)
Mn	Superoxide dismutase 3	*SOD3*	NM_001112272.1	6.05	Detoxification of reactive oxygen species	White and Scandalios (1988)
Mo	Nitrate reductase	*NAR*	X64446.1	5.07	Nitrate reduction to nitrite	Gowri et al. (1992)
Ni	Iron regulated 1 protein	*IREG1*	NM_001153299.1[a]	9.03	Plasma membrane mediated nickel transpot	Schaaf et al. (2006)
P	Inorganic phosphate transporter 1	*PHT1*	AY974041.1	5.03	Proton-coupled symport of phosphate	Nagy et al. (2006)
	Inorganic phosphate transporter 5	*PHT5*	AY974045.1	7.05	Proton-coupled symport of phosphate	Nagy et al. (2006)
	Inorganic phosphate transporter 6	*PHT6*	AY974046.1	8.06	Proton-coupled symport of phosphate	Nagy et al. (2006)
	Inorganic phosphate transporter 7	*PHT7*	AY974043.1	1.07	Proton-coupled symport of phosphate	Nagy et al. (2006)
K	Potassium channel 1	*KCH1*	Y07632.1	3.08	Potassium transport	Hoth et al. (1997)
	Potassium channel 2	*KCH2*	NM_001111650.1	6.05	Potassium transport	Philippar et al. (1999)
	Potassium channel 3	*KCH3*	NM_001111770.1	4.09	Potassium transport	Su et al. (2005)
	Potassium channel 4	*KCH4*	NM_001111691.1	3.01	Potassium transport	Büchsenschütz et al. (2005)
	Potassium channel 5	*KCH5*	NM_001112010.1	8.06	Potassium transport	Hoth et al. (1997)
Na	Sodium Bile acid symporter	*SBAS*	EU975052.1	3.05	Sodium transport	Alexandrov et al. (2009)
S	Sulfate transporter	*ST*	EU968544.1	3.04	Sulfate tranport	Takahashi et al. (2011)
	Sulfate transporter 3.4	*ST3.4*	EU957954.1	9.02	Sulfate tranport	Alexandrov et al. (2009)
Zn	Zinc transporter 10	*ZIP10*	EU973708.1	1.09	Zinc transport	Pedas et al. (2009)

[a]Homologue sequence was obtained by BLAST searches

Genome-wide association mapping of iron homeostasis in the maize association population

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Abstract

Iron (Fe) deficiency in plants is the result of low Fe soil availability affecting 30% of cultivated soils worldwide. To improve our understanding on Fe-efficiency this study aimed to (i) evaluate the influence of two different Fe regimes on morphological and physiological trait formation, (ii) identify polymorphisms statistically associated with morphological and physiological traits, and (iii) dissect the correlation between morphological and physiological traits using an association mapping population. The fine-mapping analyses on quantitative trait loci (QTL) confidence intervals of the intermated B73 x Mo17 (IBM) population provided a total of 13 and 2 single nucleotide

polymorphisms (SNPs) under limited and adequate Fe regimes, respectively, which were significantly (FDR = 0.05) associated with cytochrome P450 94A1, invertase beta-fructofuranosidase insoluble isoenzyme 6, and a low-temperature-induced 65 kDa protein. The genome-wide association (GWA) analyses under limited and adequate Fe regimes provided in total 18 and 17 significant SNPs, respectively. Significantly associated SNPs on a genome-wide level under both Fe regimes for the traits leaf necrosis (NEC), root weight (RW), shoot dry weight (SDW), water (H_2O), and SPAD value of leaf 3 (SP3) were located in genes or putative recognition sites of transcriptional regulators, which indicates a direct impact on the phenotype. SNPs which were significantly associated on a genome-wide level under both Fe regimes with the traits NEC, RW, SDW, H_2O, and SP3 might be attractive targets for marker assisted selection as well as interesting objects for future functional analyses.

6. General Discussion

Iron (Fe) efficiency can be described as the orchestration of several mechanisms including Fe sensing, uptake, allocation, and its homeostasis in the plant. The knowledge of these mechanisms was mainly derived from single gene mutant screenings (Kobayashi and Koyama, 2002). Despite the high sensitivity of this method, complex gene actions will remain undiscovered (Salt et al., 2008). Therefore, in order to complement these analyses the dissection of the genetic architecture of Fe-efficiency will be accomplished by population and quantitative genetic approaches under consideration of natural genetic variation in maize populations.

Genetic diversity and signatures of selection

An important diversity core set for maize showing a high natural allelic variation was established by Liu et al. (2003) which includes B73 and Mo17 as well as the 25 parents of the nested association mapping (NAM) population (McMullen et al., 2009). However, the determination of artificial selection of candidate genes by sequencing while using only maize genotypes would be weak. The reason for this is the weak detection power due to reduced genetic diversity and allele fixation of genes under selection caused by domestication and crop improvement processes in maize (Yamasaki et al., 2005). Therefore, the wild ancestor of maize (teosinte) was used to compare the diversity of candidate genes within both plant species in order to detect genes with selection signatures in maize.

A set of 14 genes indicating to be essential for Fe-efficiency mechanisms (Benke and Stich, 2011) was analyzed with population genetics methods using the maize core set representing a compilation of diverse genotypes (Liu et al., 2003). The majority of genes, characterized in literature as being representatives of Fe-efficiency, did not show any artificial selection as a whole. Nevertheless, 5 genes involved in the phytosiderophore (PS) synthesis, Fe remobilization, and Fe uptake were characterized by such selection signatures. The *MTK* gene, that is essential for the methionine recycling step in the PS synthesis, showed around 80% loss of nucleotide diversity in maize in comparison to teosinte. This diversity reduction was considered as significant by Tenaillon et al. (2001). Furthermore, *MTK* showed a significant reduction of the number of maize haplotypes in comparison to teosinte. In addition to that, a constellation of two statistical tests for hitchhiking effect and allele fixation indicated a recent bottleneck (Sigmon and Vollbrecht, 2010). The *NAS1* gene, an essential synthase of the precursor metabolite nicotianamine for PS in graminaceous plants, was indicated to be under directional selection according to the direct comparison to teosinte. For *NAAT1* we observed that the newly arisen polymorphisms in maize have been fixated according to the statistical test of Tajima (1989). A possible explanation for the increased diversity of maize versus teosinte for *NAAT1* and *NAS1* might be that nowadays maize is grown under more different environmental conditions than teosinte in its original habitat. Thereby, polymorphisms that were deleterious in teosinte might have become advantageous in maize. Furthermore, according to the direct comparisons of genes between maize and teosinte, we were able to detect signatures of selection for *NRAMP3* responsible for Fe remobilization from the seed vacuole during germination (Lanquar et al., 2005) and the Fe-PS complex transporter *YS1* that might be advantageous for the increased transporter-substrate affinity, which might be beneficial in the competition with microorganisms for Fe acquisition in soil (von Wirén, 1994). Finally, we were able to show that Fe-efficiency was an adaptive trait during maize domestication from teosinte. We assume that these genes played a crucial role in adaptation of maize to different environments with a varying Fe availability in the soil.

The statistical tests of population genetics used in this study focused on different demographic aspects (Flint-Garcia et al., 2009) and had different levels of statistical power to adequately detect signatures of selection. Therefore, the focus should be directed on the combination of these tests for a gene and especially their disposition should be viewed as suggestive for selection (Flint-Garcia et al., 2009). Additionally, the release of 55 million SNPs for the maize NAM founders (McMullen et al., 2009), teosinte, and *Tripsacum* by Chia et al. (2012) provides a platform to reconstruct the sequences of the most candidate genes and perform vast population genetics analyses.

The merits of population genetics are the redundancy of phenotypic information, the wealth of gene functional information, and the abundance of genotypic information for the maize core set population. However, the drawbacks refer to genes with missing functional information for Fe related processes, genes designated to be not artificially selected will be ignored, and *cis*-binding sites of non-coding regions might be missed. Therefore, to determine the contribution of these genes to Fe-efficiency, quantitative genetics might provide the necessary genetic information.

Genetic contribution to the phenotype in a hydroponic system

A high pH value, as one of the multiple abiotic effects, has a negative effect on the availability of Fe that can lead to its limitations in soil (Marschner, 1995). Besides this, other mineral nutrients might be negatively affected and therefore causing a deficiency phenotype without a clear element affiliation. However, to get a clear phenotype depending only on the Fe level in the rhizosphere, the hydroponic system promises to be the method of choice (Cook et al., 2012) and was used in my studies (Benke et al., 2014, in preparation).

In preliminary experiments to determine the Fe-efficient and inefficient phenotype in maize, we observed that B73 was the most Fe-efficient genotype at the low Fe regime, whereas Mo17 was characterized as being most susceptible (Fig. 1). Compared to Mo17, the B73 inbred line showed obvious

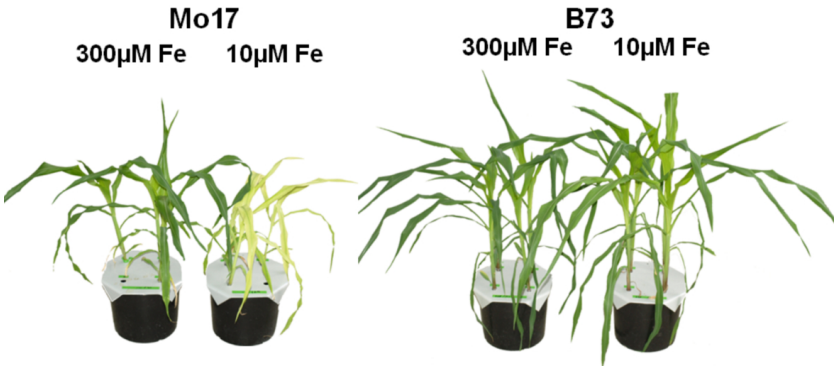

Figure 1: Phenotypic comparison between Mo17 and B73 growing at the low (10μM Fe) and high (300μM Fe) iron regime.

phenotypic superiority in biomass production, root architecture, and relative chlorophyll content. Furthermore, mineral nutrient analyses of the dry shoot material revealed a higher accumulation of cadmium and aluminum in Mo17 in comparison to B73 under the low Fe regime. This indicated that Mo17 has not only to cope with Fe deficiency but also with higher concentration of toxic minerals. Moreover, the Fe concentrations in leaves of both genotypes were similar, indicating that B73 uses Fe more efficiently than Mo17.

Due to their differences in Fe-efficiency, the inbred lines Mo17 and B73 present the required prerequisites for the construction of a segregating population which might aid to dissect the chromosomal locations of QTLs causing the Fe-efficient phenotype.

Using intermated recombinant inbred lines (IRILs) for QTL mapping

A segregating population was developed by Lee et al. (2002) crossing the parental inbred lines B73 (Fe-efficient) and Mo17 (Fe-inefficient). However, the main advantage of this population is the high amount of recombinations resulting from the four cycles of intermating before the F_2 generation. This provided a finer mosaic of the chromosomal segments compared to non-intermated recombinant inbred lines and increased the resolution of QTL mapping (Darvasi and Soller, 1995; Balint-Kurti et al., 2007). The provided set of 94 IRILs has nearly the same resolution as 300 - 350 recombinant inbred lines created without any intermating steps (Coe and Schaeffer, 2005).

QTL mapping to dissect genetic architecture in the IBM population

The available Fe in the rhizosphere and in the plant has an immense influence on morphological and physiological trait formation (Marschner, 1995). At the low Fe regime, we observed a moderate to high influence of individual QTLs on the phenotype variation, in comparison to the high Fe regime. One explanation could be a low genetic complexity which increases the probability to identify in these QTL confidence intervals putative essential genes (Waters and Grusak, 2008) contributing a high effect to the Fe homeostasis.

The gene family most often detected within the QTL confidence intervals at the low Fe regime for the traits relative chlorophyll content, the root architecture, and leaf necrosis was of the metal transporter protein family (*MTP*). In fact, under Fe deficiency, the Fe uptake mechanisms are enhanced to fulfill the demand for Fe in plants (Schaaf et al., 2004). However, other metals besides Fe might accumulate and increase the Fe stress symptoms (Marschner, 1995). The *MTP* is involved in the detoxification of metals accumulated in

the cytoplasm (Hanikenne et al., 2005; Talke et al., 2006). The MTP family provides the prerequisite function for metal tolerance which becomes essential during Fe limitation in the plant. An efficient MTP mechanism might avoid the metal accumulation and prevent deleterious metal interactions with the Fe binding sites in the cytoplasm. Therefore, further analysis with the *MTP*s might be essential for Fe-efficiency studies which have so far not been considered as relevant under both, low or high Fe concentrations.

We observed that low and high Fe concentrations influenced the morphology and physiology of maize growing in a hydroponic system (Benke et al., 2014, in preparation). Furthermore, it is known that a low Fe concentration causes an increased accumulation of mineral nutrients other than Fe that might have deleterious effects on maize development (Thoiron et al., 1997; Meda et al., 2007; Kanai et al., 2009). However, a high Fe concentration displaced several mineral nutrients. Therefore, the genetic contribution to the regulation, accumulation, and leaf tissue mineral nutrients composition that are crucial for metabolism and development (Lowry et al., 2012) of maize growing under different Fe regimes need to be examined. The measured mineral nutrient concentrations of the dried shoot material were used for QTL detection. These analyses provided essential QTLs for Cd detoxification (*HMA3*, *ZIP4*), transporters (*ZIP10*, *NRAMP2*) of mineral nutrients or toxic metals, and oxidative stress response (glutaredoxin). The most interesting gene, namely *PHT1;5* was comprised within a phosphorus QTL confidence interval detected under low Fe regime (Benke et al., in preparation). The role of phosphorus becomes more important during Fe deficiency where phosphorus negatively affects the Fe availability in chlorotic leaves due to precipitation (Marschner, 1995). Therefore, the homeostasis of phosphorus is essential in maize particularly under Fe deficiency. *PHT1;5* might contribute to phosphorus depletion and therefore becomes essential for an Fe-efficient performance. However, a functional proof of *PHT1;5* is still lacking in maize.

QTL mapping provides the genetic map position of QTLs and gives an

essential hint for chromosomal regions responsible for trait variation. However, the confidence intervals of the QTLs still comprise hundreds of genes corresponding to millions of base pairs on the physical map (Flint-Garcia et al., 2005). A further limiting factor is the bi-parental cross itself that allows only the differentiation between two alleles at a locus. As an alternative method, association mapping can be applied to fine-map the genes in QTL confidence intervals. Genome-wide association mapping will contribute to the dissection of the genetic architecture of Fe-efficiency within an association mapping maize population including a high allelic diversity (Flint-Garcia et al., 2005).

Using the maize association population for association mapping

The determination of a highly diverse maize germplasm set which covers the maximum allele richness was performed using 94 SSR markers by Liu et al. (2003) for 260 maize genotypes representing 82% of the worldwide maize allele diversity. Furthermore, Flint-Garcia et al. (2005) presented the association maize population of 302 genotypes including a large proportion of the 260 genotypes of Liu et al. (2003). Furthermore, this population provides a high resolution and high statistical power platform for association analyses (Flint-Garcia et al., 2005; Cook et al., 2012). Moreover, the association population represented a public germplasm used for breeding programs worldwide that include genotypes that were collected from temperate as well as from tropical regions (Flint-Garcia et al., 2005). These genotypes might be differently adapted to varying Fe availabilities in their originating locations (Benke and Stich, 2011). Furthermore, in several locations special genotypes are required adapted to low Fe availability which might include alleles responsible for an Fe-efficient management in maize. The detection of such alleles would be beneficial for the determination of mechanisms involved in Fe use management. Therefore, we applied two Fe regimes in a hydroponic

system to evaluate the genetic influence on the phenotype formation. The association population showed under low and high Fe regimes a quantitative phenotypic variation with moderate to high broad sense heritabilities. This finding indicates that the data of our study provide a powerful basis for detecting QTLs for morphological and physiological performance under different Fe regimes (Benke et al., in review).

Prerequisites to reduce spurious associations

Association mapping analyses require knowledge of the population structure to reduce spurious marker-phenotype associations (Flint-Garcia et al., 2003). The population structure was examined for the maize association population based on 89 SSR markers by Flint-Garcia et al. (2005) with the STRUCTURE software (Pritchard et al., 2000) and further used for the association analyses.

As a further statistical method to reduce the false positive and negative associations, calculation of relatedness was suggested for consideration in the association analyses (Yu et al., 2006; Kang et al., 2008). Because of the lower price and the higher availability of single nucleotide polymorphism (SNP) compared to simple sequence repeats (SSR) (Hamblin et al., 2007; Astle and Balding, 2009), the relatedness calculation was performed with SNPs evaluated for the association mapping population. Kang et al. (2008) suggested to calculate a simple identical-by-state allele-sharing kinship matrix which reduces false associations more effectively than the kinship matrices generated by previous methods (Yu et al., 2006). Therefore, in my study the kinship matrix was calculated according to Kang et al. (2008) and used in the association analyses.

The use of the kinship matrix **K** in association analyses allows to describe the relation of smaller groups or even between two individuals. Furthermore,

population structure matrix **Q** describes remote ancestry and assigns the individuals to large groups (Astle and Balding, 2009). Therefore, the Q + K approach of Yu et al. (2006) is able to account for multiple relation constellations among individuals and, thus was used in the frame of this thesis for association analyses.

Association mapping to dissect Fe-efficiency

The complex trait Fe-efficiency was separated into 13 traits to expand the coverage of Fe affected sites that consequently might be coded by diverse genes. Furthermore, these genes might be linked to marker-phenotype association that need to be detected according to the genome-wide association mapping approach (Oraguzie and Wilcox, 2007). The single marker-phenotype association performed for 287,390 SNPs over 267 genotypes revealed in total 18 and 17 significant SNP markers (FDR = 0.05, multiple test correction) for low and high Fe regime, respectively. The number of traits that contributed to significant SNPs detection for each Fe regime was three. That was lower than detected with linkage mapping analyses (Benke et al., 2014). This might be due to the fact that the multiple test correction underlying a vast amount of markers was very stringent, which in consequence allows only the detection of highly significant SNPs (Cook et al., 2012). Moreover, the detected significant SNPs located in these traits were linked mostly to regulative elements like E3 ubiquitin-protein ligase EL5 indicating involvement in maintenance of cell viability (Koiwai et al., 2007) during the remobilization of Fe from source leaves during limitation in the sink organs. Furthermore, mitogen-activated protein kinase 8 might be important for stress response during mineral nutrient displacement like phosphorus under high Fe concentrations in maize (He et al., 1992; Pan et al., 2012). Besides the known regulative proteins linked to significant associated SNPs, several significant SNPs remained unlinked to a gene. These unknown SNPs might indicate putative *cis*-recognition sites for transcriptional regulators (Stam et al., 2002) and have an essential effect on

the translation initiation. Functional analyses especially under different Fe regimes on these binding sites would provide insights on their mode of action.

Furthermore, the association mapping approach was used for confirmation and fine-mapping of QTLs detected in the linkage mapping analyses. The fine-mapping of QTL confidence intervals revealed an obviously lower phenotypic variation explained by the significant SNPs compared to the SNPs detected by genome-wide associations. An explanation might be that the variation of detected QTLs was overestimated in the linkage analyses due to a limited sample size (Utz et al., 2000) covering a limited amount of recombinations in the bi-parental populations. This would indicate that the linkage mapping provided confidence intervals comprising genes of small effects. Nevertheless, these genes contribute to a more detailed genetic architecture and might resolve the complexity of Fe-efficiency. However, validation of these alleles need to be performed before using them in breeding programs.

Validation of genes and chromosomal loci detected to be involved in Fe-efficiency process

Genes related to Fe-efficiency under artificial selection or genes detected by association analyses were detected through statistical methods and need functional confirmation. An appropriate method is transposon tagging, in which an Fe-efficient genotype needs to be crossed with a transposon inducing maize line in order to generate an Fe-inefficient genotype. The transposon insertion needs to be identified and characterized in the genes of the inefficient genotype (Curie et al., 2001; Brutnell, 2002), which requires thousands of plants for such screens. The silencing of genes according to interfering RNA (RNAi) is a direct proof of the gene influence on the Fe-efficient phenotype (Miki and Shimamoto, 2004). Furthermore, the determination of gene influence is feasible according to analyses of genotypes with targeting-induced local lesions in genomes (TILLING) (Till et al., 2004).

Near isogenic lines are widely used for validation of alleles previously identified in linkage or association mapping. The near isogenic lines contain a small genomic region of an Fe-efficient line which was introgressed into an Fe-inefficient isogenic background. An alternative to the near isogenic line is to focus on the detected quantitative trait loci involved in the phenotype variation to select by investigating QTL isogenic recombinants (QIRs). The method for selection of QIRs was described by Peleman et al. (2005), in which a QIR plant needs a recombination event in one QTL, while these are homozygous at all other QTLs for the same trait.

Conclusions and outlook

The use of population genetic methods allowed the detection of artificial selection in genes involved in Fe related processes which indicates their essential role in the adaptation of maize to varying Fe availabilities in several environments. The results of linkage mapping provided a vast amount of QTLs that allow to dissect the complex trait Fe-efficiency according to 13 traits that are related to the Fe concentration in the plant. In addition to the detected QTLs responsible for Fe homeostasis, the QTLs detected for the mineral nutrient concentration in the plant suggested to be important for the mineral nutrients homeostasis under different Fe regimes. Furthermore, association mapping analyses provided regulative elements explaining a high phenotypic variation at both the low and high Fe regime. SNPs detected beyond coding regions of genes might be important *cis*-binding-sites for transcription factors. The fine-mapping results of the QTL confidence intervals suggested the presence of small effect genes that was in an inflationary contrast to the linkage mapping analyses using a limited sample size. The marker-trait associations and QTLs identified by linkage mapping detected for Fe related traits are promising for breeding programs in order to improve Fe use efficiency in maize. Furthermore, an efficient Fe accumulation in veg-

etative organs and an improved Fe homeostasis and remobilization might be an important prerequisite for studies dealing with Fe biofortification.

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7. Summary

Maize is susceptible to severe Fe-deficiency symptoms when growing on soils with high pH. Therefore, development of Fe-efficient maize genotypes would aid to overcome Fe limitation on these soils. However, Fe-efficiency is a quantitative trait depending on complex mechanism interactions. The determination of these mechanisms would provide a better understanding of the complex trait Fe-efficiency. In the actual study, the determination of Fe-efficiency involved mechanisms were tackled by population and quantitative genetics. In fact, population genetics facilitate the discovery of genes being important to crop improvement based on a comparison of gene evolution and its ancestral genetic material. Linkage mapping and association analyses require both phenotypic variation and polymorphic markers to determine important quantitative trait loci (QTL). The objective of this research was to dissect the genetic architecture of Fe-efficiency in maize by applying different genetic approaches.

Artificial selection during domestication and (or) crop improvement can result in limitation of sequence variation at candidate genes that could limit their detection by quantitative genetic approaches. The objectives of our study were to (i) describe patterns of sequence variation of 14 candidate genes for mobilization, uptake, and transport of Fe in maize, as well as regulatory function and (ii) determine if these genes were targets of selection during domestication. This study was based on 14 candidate genes sequences of 27 diverse maize inbreds, 18 teosinte inbreds, and one *Zea luxurians* strain as an outgroup.

The experimental results suggested that the majority of candidate genes for Fe-efficiency examined in this study were not target of artificial selection. Nevertheless, the genes *NAAT1, NAS1,* and *MTK* coding for enzymes involved in phytosiderophore production, *NRAMP3* responsible for Fe remobilization during germination, and *YS1* transporting PS-Fe-complexes into the root showed signatures of selection. These genes might be important for the adaptation of maize to diverse environments with different Fe availabilities. This in turn suggests, that Fe-efficiency was an adaptive trait during maize domestication from teosinte.

Identification of QTL provides information on the chromosomal locations contributing to the quantitative variation of complex traits. The benefit of QTL mapping compared to mutant screenings is the possibility to detect multiple genes which may be associated with the phenotypic trait. The objectives of our studies were to (i) identify QTLs for morphological and physiological traits related to Fe homeostasis, (ii) analyze Fe-dependent expression levels of genes known to be involved in Fe homeostasis as well as positional candidate genes from QTL analysis, and (iii) identify QTLs which control the mineral nutrient concentration difference. Our studies were based on experimental data of 85 genotypes from the IBM population cultivated in a hydroponic system.

The QTL mapping of morphological and physiological traits provided new putative candidate genes like Ferredoxin 1, putative ferredoxin *PETF*, *MTP4*, and *MTP8* which complement the genes already known as being responsible for efficient Fe homeostasis at both, deficient and sufficient Fe regime. Furthermore, the candidate gene expression indicated a *trans*-acting regulation for *DMAS1, NAS3, NAS1, FDH1, IDI2, IDI4,* and *MTK*. The mineral element trait QTL confidence intervals comprised candidate genes that sequestrate Cd in vacuoles (*HMA3*), transport Fe^{2+} into the root cells (*ZIP10*), protect the cell against oxidative stress (glutaredoxin), ensure micro nutrient homeostasis during sufficient iron regime (*NRAMP2*), regulate

protein activities (*PP2C*), and prevent deleterious accumulation and interaction of specific elements within cells (*PHT1;5*, *ZIP4*).

Association mapping is promising to overcome the limitations of low allele diversity and absent recombinations events causing poor resolution in detecting QTL by linkage mapping. In order to unravel the genetic architecture of Fe-efficiency a vast association mapping panel comprising 267 maize inbred lines was used to (i) detect polymorphisms affecting the morphological/physiological trait formation and (ii) fine map QTL confidence intervals determined according to linkage mapping.

Some of the SNPs located beyond coding regions of genes that might be important *cis*-binding-sites for transcription factors. Furthermore, genes detected at the Fe-deficient regime indicate to be involved in universal stress response. However, genes linked to SNPs detected at Fe-sufficient regime might comprise alleles of Fe inefficient genotypes causing inferior trait expression.

The combination of several approaches provided a valuable resource of candidate genes which might aid to increase our understanding of the mechanisms of Fe-efficiency in maize and foster the efforts in breeding superior cultivars by applying molecular marker techniques.

8. Zusammenfassung

In den Anbaugebieten, wie Nebraska und dem ariden/semiariden Gebieten von den Great Plains, die charakteristisch für kalkreiche Böden und hohen pH sind, ist Eisen-(Fe)-Mangel ein agronomisch wichtiges Problem, das Ernteausfälle und Qualitätsminderungen in der Kulturpflanze Mais verursacht. Aus diesem Grund ist die Entwicklung von Fe-effizienten Mais Sorten notwendig, um diese limitierende Böden für den Maisanbau zu erschließen. Allerdings ist die Eigenschaft für Fe-Effizienz quantitativ und basiert auf komplexe Wechselwirkungen zwischen der Gesamtheit von Mechanismen. Es liegt auf der Hand, dass eine Ermittlung dieser Mechanismen ein besseres Verständnis für die Fe-Effizienz als ein komplexes Merkmal darlegen würde. In der aktuellen Studie lag der Fokus auf der Ermittlung von Fe-Effizienz bezogenen Mechanismen, welche mittels populations- und quantitativgenetischen Verfahren detektiert werden sollten. Fakt ist, dass die Populationsgenetik die Gensequenzenvergleiche der entscheidenden Gene, welche unter dem Selektionsdruck der Züchtungsvorgänge in der Kulturpflanze eine entscheidende Verbesserung brachten, zwischen Elitematerial und Maisvorfahren zur Detektion nutzt. Sowohl die Analyse von kodierenden Genloci für quantitative Merkmale (QTL) als auch die Assoziationskartierung erfordern phänotypische Variation und polymorphe Marker, um genetisch beeinflussende Regionen eingrenzen zu können. Das Hauptaugenmerk der vorliegenden Arbeit richtete sich auf die genetische Architektur von Fe-Effizienz in Mais und auf die Untersuchung der Anwendung von unterschiedlichen genetischen Methoden.

Die Domestikation und Verbesserung der Kulturpflanze kann durch eine gerichtete Selektion zu Diversitätslimitierung in Kandidatengenen führen, deren Detektion anhand von quantitativen genetischen Ansätzen gemindert werden könnte. Die Ziele unserer Studie waren (i) die Beschreibung der Sequenzdiversität in 14 Kandidatengenen mit der Eigenschaft sowohl für Mobilisierung, Aufnahme und Transport von Fe in Mais als auch für regulative Funktion sowie (ii) zu untersuchen, ob diese Gene gezielt während der Domestikation von Teosinte selektiert wurden. Diese Studie wurde mit Sequenzen von 14 Kandidatengenen, die sowohl in 27 diversen Mais und 18 Teosinte Inzuchtlinien als auch in einem entfernt verwandtem Zea luxurians Stamm sequenziert wurden, durchgeführt.

Die Versuchsergebnisse dieser Studie deuteten darauf hin, dass die Mehrheit der Kandidatengene für Fe-Effizienz nicht das Ziel der Selektion war. Nichtsdestotrotz zeigten Gene wie *NAAT1, NAS1* und *MTK*, die für Enzyme der Phytosiderophore (PS) Synthese kodieren, *NRAMP3*, das für die Fe Remobilisation während der Keimung verantwortlich ist und *YS1*, das für den Transport von Fe-PS-Komplexen in der Wurzel sorgt, Anzeichen von gerichteter Selektion. Diese Gene könnten für die Anpassung von Mais, der an verschiedenen Standorten mit variierenden Fe Verfügbarkeiten angebaut wird, von Bedeutung sein. Das wiederum bedeutet, dass Fe-Effizienz ein angepasstes Merkmal während der Domestizierung von Teosinte zu Mais war.

Die Identifikation von QTL erlaubt die Eingrenzung von chromosomalen Regionen, welche zu der quantitativen Variation von komplexen Merkmalen beitragen. Der Vorteil von der QTL Kartierung gegenüber Mutanten basierten Analysen ist die Möglichkeit mehrere Gene gleichzeitig zu detektieren, welche mit dem phäno-typischen Merkmal in Verbindung gebracht werden können. Die Ziele unserer Studien waren (i) die Identifikation von QTL für morphologische und physiologische Merkmale, die für Fe-Homöostase evaluiert wurden (ii) Analyse von Fe basierenden Genexpression von Genen, die sowohl bekannt für deren Involvierung in der Fe-Homöostase sind als auch durch die positionelle Lokalisation mittels der QTL Analyse sowie (iii) die

Identifikation von QTL, welche die Konzentration der mineralischen Nährstoffe kontrollieren. Diese Studien basierten auf Experimentdaten, welche für 85 Genotypen der IBM Population mittels einem hydroponischem System evaluiert worden sind.

Die QTL Kartierung, die mittels morphologischer und physiologischer Merkmale evaluiert werden konnten, lieferten potentielle neue Kandidaten Gene wie Ferredoxin 1, ein potentielles Ferredoxin Gen *PETF*, sowie *MTP4* und *MTP8*. Diese Gene haben eine unterstützende Wirkung auf bereits bekannte Gene, die verantwortlich für eine effiziente Fe-Homöostase unter niedriger und hoher Fe Behandlung sind. Des Weiteren wurde beobach-tet, dass die Gene *DMAS, NAS1, NAS3, FDH1, IDI2, IDI4* und *MTK* eine trans-gerichtete Regulation aufwiesen. Die Vertrauensintervalle von QTL, welche für die mineralischen Konzentrationsmerkmale detektiert wurden, grenzten Kandidaten Gene ein, welche Cd in Vakuolen sequestrierten (*HMA3*), Fe^{2+} in die Wurzelzellen transportieren (*ZIP10*), die Zelle vor oxidativen Stress bewahren (Glutaredoxin), die Miktonährstoff-Homöostase unter ausreichender Fe Zufuhr gewährleisten (*NRAMP2*), die Proteinaktivität regulieren (*PP2C*) sowie die Zelle vor schädlicher Akkumulation und Interaktion von speziellen Nährstoffen schützen (*PHT1;5*, *ZIP4*).

Die Assoziationskartierung verspricht die Einschränkungen, die bei der Kopplungskartierung eine geringe Diversität und fehlende Rekombination zur niedrigen genetischen Auflösung in der QTL Detektion führen, zu reduzieren. Um die genetische Architektur von Fe-Effizienz zu ermitteln, wurde ein breit aufgestelltes Assoziationskartierungsset eingesetzt, das 267 Mais Inzuchtlinien beinhaltet, um (i) Polymorphismen zu detektieren, welche die morphologischen/physiologischen Merkmalsausprägungen beeinflussen sowie (ii) QTL Vertrauensintervallen, die mit der Kopplungskar-tierung detektiert wurden, feinkartieren.

Einige der SNPs, die jenseits der kodierenden Genregionen lokalisiert wurden, könnten wichtige cis-bindende Regionen für Transkriptionsfaktoren

sein. Des Weiteren weisen die detektierten Gene unter der niedrigen Fe Behandlung hin, dass sie in eine universelle Stressantwort involviert sind. Allerdings könnten die SNP gekoppelten detektierten Gene unter der hohen Fe Behandlung Fe-ineffiziente Allele tragen, welche einen Nachteil in der Merkmalsausprägung verursachen könnten.

Die Kombination der jeweiligen Methoden lieferte eine wertvolle Anzahl von Kandidaten Genen, welche unser Verständnis für Fe-Effizienz Mechanismen in Mais unterstützen sollen und die Bemühungen in der Kulturpflanzenzüchtung mittels Anwendung von molekularen Marker Techniken fördern.

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Halberstadt, 13.10.2014

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