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DISSERTATION

NUTRITIONAL REGULATION OF DNA METHYLATION AND GENE EXPRESSION IN MAIZE

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“The love for all living creatures is the most noble attribute of man.”

— Charles Darwin

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List of Abbreviations

A	Adenine
AGO	Argonaute
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BGI	Beijing Genomics Institute
bp	Basepair
C	Cytosine
$Ca(NO_3)_2$	Calcium nitrate
$CaSO_4$	Calcium sulfate
CH_3	Methyl group
CMT	Chromomethylase
CO_2	Carbon dioxide
Ctrl	Control
DAG	Diacylglycerol
DEG	Differentially expressed gene
DME	Demeter
DMG	Differentially methylated gene
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DRM	Domains rearranged methyltransferase
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
FPKM	fragments per kilobase of transcript per million mapped reads
G	Guanine
GS-GOGAT	Glutamine synthase-Glutamine oxoglutarate aminotransferase

H	Any base but guanine
H_2O_2	Hydrogen peroxide
KH_2PO_4	Potassium dihydrogen phosphate
Log_2FC	Binary logarithm fold change
maizeGDB	Maize Genetics and Genomics Database
MET	Methyltransferase
MspI	Restriction enzyme cutting at C|CGG
N	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NAS	Nicotianamine synthase
NRT	Nitrate transporter
nt	Nucleotide
OD	Optical density
P	Phosphorus
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEPC	Phosphoenolpyruvate carboxylase
PFD	Photosynthetically active photon flux density
PHT	Phosphate transmembrane transporter
ppm	Parts per million
RdDM	RNA-directed DNA methylation
RDR	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNA pol	RNA polymerase
RNA-seq	RNA-sequencing

ROS	Repressor of silencing
RRBS	Reduced representation bisulfite sequencing
RRG	Reduced representation genome
siRNA	Small interfering RNA
SPS	Sucrose-phosphate synthase
SPX domain	SYG1/Pho81/XPR1 proteins domain
T	Thymine
TE	Transposable element
TSS	Transcription start site
UDP	Uridine diphosphate
UV-VIS	Ultraviolet-visible
ZIP	Zinc transporter / Zrt/Irt-like protein
Zn	Zinc
$ZnSO_4$	Zinc sulfate
$MgCl_2$	Magnesium chloride
H_3BO_3	Boric acid
$MnSO_4$	Manganese sulfate
$CuSO_4$	Copper sulfate
K_2SO_4	Potassium sulfate
$(NH_4)_6Mo_7O_{24}$	Ammonium heptamolybdate

Abstract

DNA methylation in plants plays a role in transposon silencing, genome stability and gene expression regulation. Environmental factors alter the methylation pattern of DNA and recently nutrient stresses, such as phosphate starvation, were shown to alter DNA methylation. DNA methylation had been frequently addressed in plants with notably small genomes that are poor in transposons. Here, part of the DNA methylome of nitrogen-, phosphorus- and zinc-deficient (-N, -P and -Zn, respectively) maize roots were compared by reduced representation sequencing and their relationship with gene expression under prolonged stresses analyzed. Tremendous DNA methylation loss was encountered in maize under nitrogen and zinc deficiency, but much less under phosphorus deficiency. This occurred only in the symmetrical cytosine contexts, predominantly in CG context, but also in the CHG context. In contrast to other plants, differential methylation in the more flexible CHH context was essentially absent. For each sample, specific nutrient deficiency-regulated genes were differentially expressed. In -Zn samples the lowest number of differentially expressed genes was found while -N and -P samples contained a similar number of differentially expressed genes. For all samples, differentially methylated regions (DMRs) were predominantly identified in transposable elements (TEs). A minor fraction of such DMRs was associated with altered gene expression of nearby genes in -N and -P. Interestingly, although these TEs were mostly hypomethylated, they were associated with both up- and down-regulated gene expression. For -Zn, these associations were not found but a correlation between hypomethylation of gene bodies and expression of some genes. Here again, hypomethylation occurred with up- and downregulation of gene expression. The results suggested a different methylome regulation in maize

compared to rice and *Arabidopsis* upon nutrient deficiencies indicating a nutrient- and species-specific association of genomic DNA methylation and gene expression.

The limited correlation between differential DNA methylation and gene expression suggested that heritable regulation of the expression of nutrient deficiency-regulated genes was not the primary function of the methylation loss. Rather, the major function of the DNA methylation loss in this experiment may have been to increase the genetic diversity in the next generation by increased frequency of recombination events, mutations and transposable element movements.

Zusammenfassung

DNA Methylierung spielt in Pflanzen eine wichtige Rolle für die Stilllegung von Transposons, für die Genomstabilisierung sowie bei der Regulation der Genexpression. Umweltfaktoren ändern das Methylierungsmuster und inzwischen wurde gezeigt, dass auch Nährstoffstress, wie zum Beispiel Phosphatmangel, das Methylierungsbild ändern kann. DNA Methylierung wurde häufig in Pflanzen mit besonders kleinem Genom und einem geringfügigen Anteil an Transposons untersucht. Hier wurde ein Teil des DNA Methyloms von Maiswurzeln mit Nitrat-, Phosphat- oder Zinkmangel (-N, -P bzw. -Zn) mittels ‚reduced representation' Sequenzierung miteinander verglichen und der Zusammenhang mit der Genexpression unter andauerndem Stress analysiert. Unter Nitrat- und Zinkmangel war ein starker Verlust von DNA Methylierung zu verzeichnen, unter Phosphatmangel jedoch nur eine weit schwächere Minderung. Der Verlust trat in den symmetrischen Kontexten des Cytosins auf, vor allem im CG Kontext, aber auch im CHG Kontext. Im Unterschied zu anderen Pflanzen, war eine differenzielle Methylierung im flexibleren CHH Kontext quasi nicht vorhanden. Bei jeder Behandlung lag typische Nährstoffmangel-regulierte differenzielle Genexpression vor. Die niedrigste Anzahl an differenziell exprimierten Genen wurde unter -Zn gefunden, während -N und -P Behandlungen etwa gleich viele differenziell exprimierte Gene aufwiesen. In allen Proben lagen die meisten differenziell methylierten Regionen (DMRs) in Transposons (TEs). Bei einem kleinen Teil der DMRs gab es nahegelegene differenziell exprimierte Gene in -N und -P. Obwohl die meisten der differenziell methylierten TEs hypomethyliert waren, war die Expression der nahen Gene sowohl hoch- als auch runterreguliert. In -Zn gab es keinen signifikanten Zusammenhang zwischen differenziell methylierten TEs und der Expression nahegelegener Gene. Es wurde

jedoch ein Zusammenhang zwischen Hypomethylierung einiger Gene und deren Expression gefunden, die auch hier teils hoch-, teils runterreguliert waren. Die Ergebnisse wiesen auf eine andere Methylom-Regulierung bei Nährstoffmängeln in Mais als in Reis und *Arabidopsis* hin und verwiesen auf eine nährstoff- sowie speziesabhängige Anpassung der genomischen DNA Methylierung im Zusammenhang mit der Genexpression.

Die schwache Korrelation zwischen differenzieller Methylierung und Genexpression deutete darauf hin, dass eine vererbbare Regulation der Expression von Nährstoffmangel-regulierten Genen nicht die Hauptfunktion der Änderungen im Methylierungslevel war. Vielmehr könnte dem Verlust der Methylierung eine größere Rolle in der Erhöhung der genetischen Diversität in der nächsten Generation durch Zunahme von Rekombinationsereignissen, Mutationen und Bewegung von Transposons zukommen.

1 Introduction

1.1 The importance of plant nutrition

All plants need a range of different nutrients for their survival and growth. Among the essential nutrients there are macro- and micronutrients, depending on how much of each nutrient is needed by the plants. Plants contain profound adaptation strategies that influence many aspects of growth, development and metabolism, when essential nutrients are insufficiently available. Without sufficient nutrient supply crop plants cannot provide full yield. Nitrogen (N) and phosphorus (P) belong to the most limiting nutrients for proper plant development and high yield. But with a growing world population, most efficient use of crops becomes more and more inevitable. Additionally, a great part of soils used for agriculture does not contain a sufficient amount of essential nutrients. Therefore, many soils are extensively fertilized posing ecological threats and a waste of resources, especially with phosphorus being a finite resource. Plants also suffer strongly from zinc deficiency and it is estimated that about 50% of cereal crop agricultural soils are potentially zinc-deficient (Cakmak, 2011; Nielsen, 2012). But not only plants need a sufficient amount of zinc (Zn), also animals and humans are affected by a deficiency. Among humans, zinc deficiency concerns over 2 billion people worldwide with a range of health impacts (Mocchegiani et al., 2013; Prasad, 2008). In respect to that, sufficient intake of zinc via crop products helps alleviate this difficulty. One approach for achieving this is biofortification to increase the nutritional value of important crop plants. As plants are tremendously important for all living organisms and provide the basis of human nutrition, a lot of research has been done for decades to unravel the mechanisms of how plants develop under usage of nutrients or how they cope with nutrient deficiencies. Through the obtained knowledge

a lot has been achieved already in breeding high yielding and nourishing crop plants. Despite all research, there remain big gaps in the knowledge about many plant functions, including the influence of epigenetic mechanisms on nutritional coping processes.

1.2 The roles of nitrogen, phosphorus and zinc in plants

Two of the most important and most limiting macronutrients are nitrogen and phosphorus. Both are important components of DNA and RNA. Additionally, nitrogen is part of amino acids, chlorophyll and some relevant plant hormones (Yang et al., 2015) while phosphorus is a necessary player in photosynthesis and thereby influencing carbohydrate content as well as playing a role in energy provision as part of ATP and as a structural element in phospholipids (Wu et al., 2003; Zhang et al., 2014). P is involved in the control of key enzyme reactions and deficient P has important consequences on the respiratory metabolism. Only few soils contain plant available phosphorus in sufficient amounts (Schachtman et al., 1998; Theodorou and Plaxton, 1993). Zinc is a significant micronutrient and an important cofactor for a high number of transcription factors and enzymes in plants. As a catalytic component, it enables or enhances the reactions performed by the enzymes. Zinc can function as structural component aiding in appropriate protein folding. Additionally, it is needed for proper membrane integrity and takes part in RNA and DNA metabolism as well as gene expression regulation. It is further involved in detoxification of superoxide radicals and synthesis of phytohormones (Assunção, Herrero, et al., 2010; Assunção, Schat, et al., 2010; Broadley et al., 2011; Yamaji et al., 2013).

1.3 Nutrient-specific gene expression under deficiency conditions

When a plant suffers from deficiency of a nutrient, usually high affinity transporters for the lacking nutrient are expressed in higher number which increases most efficient uptake and use of any remaining amount of the particular nutrient.

During nitrogen deficiency, a large number of genes are differentially regulated compared to well-supplied conditions. Several genes have been identified as highly consistently upregulated in all plants and thus can serve as nutrient-specific markers for individual deficiencies. These include some high affinity nitrate transporter genes (NRTs) of class II, which accumulate at low nitrate (Schluter et al., 2012; Yang et al., 2015). Additionally, carbohydrates (sugars, starch) accumulate in N deficiency, which might result from reduced carbon demand and decreased sink strength in the plant and alters expression of genes involved in carbohydrate metabolism (Boussadia et al., 2010; Comadira et al., 2015; Schluter et al., 2012). Nitrate reductases are rapidly decreased under low nitrate, as these enzymes reduce nitrate to nitrite and under reduced nitrate supply are not needed (Menz et al., 2016; Schluter et al., 2012). Glutamate-ammonia ligase (= glutamine synthetase) and Glutamine oxoglutarate aminotransferase, which together build the GS-GOGAT-pathway, are important for nitrogen assimilation and are often found to be either unchanged or upregulated during nitrogen deficiency in plants (Comadira et al., 2015; Schluter et al., 2012).

In plants suffering from phosphorus deficiency, high-affinity transporters like the inorganic phosphate transmembrane transporters (PHTs) are more highly expressed which enables them to most efficiently take up small amounts of remaining phosphorus (Li et al., 2012; Secco et al., 2015). Acid phosphatases are shown to be consequently upregulated under P stress in various plants for an increased P uptake (Aono et al.,

2001; Li et al., 2012; Secco et al., 2015; Vance et al., 2003; Wang et al., 2014; Wu et al., 2003; Yong-Villalobos et al., 2016; Zhang et al., 2014; Zörb and Müller, 2015). Phosphoenolpyruvate carboxylase (PEPC) often seems to be P deficiency-responsive, even though there seem to be big differences between plant species and/or plant tissues. So it was shown that PEPC increased in cluster roots of white lupin (*Lupinus albus*) as well as in roots and shoots of chickpea (*Cicer arietinum*), in oilseed rape cell cultures and in *Sesbania rostrate* (Aono et al., 2001; HOFFLAND et al., 1992; Moraes and Plaxton, 2000; Vance et al., 2003; Wang et al., 2014). By contrast, PEPC expression was reduced in maize leaves and in *Arabidopsis thaliana* under P deficiency (Wu et al., 2003; Zhang et al., 2014).

Among the genes known to be upregulated under zinc deficiency are zinc transporters from the ZIP (Zrt/Irt-like Proteins) family, especially ZIP1 and ZIP2. Increasing the amount of these transporters facilitates uptake of traces of zinc (Assunção, Herrero, et al., 2010; Assunção, Schat, et al., 2010; Grotz et al., 1998; Van De Mortel et al., 2006). Carbonic anhydrase, which catalyzes CO_2 hydration, requires Zn and its transcripts were shown to be reduced under Zn deficiency in spinach and rice plants (Broadley et al., 2011; Randall and Bouma, 1973). Nicotianamine synthase (NAS), especially NAS4, is upregulated during zinc deficiency (Assunção, Schat, et al., 2010). NAS synthesizes nicotianamine, which in turn is involved in uptake and transportation of heavy metals, as for example zinc and iron (Bonneau et al., 2016), therefore Zn uptake is increased by upregulated NAS expression. In contrast, alcohol dehydrogenase, which catalyzes reduction of acetaldehyde to ethanol and thereby regenerating NAD^+, is downregulated in zinc-deficient plants (Broadley et al., 2011; Magonet et al., 1992) (Broadley et al., 2011; Magonet et al., 1992). A downregulation is also experienced regarding superoxide dismutases, which detoxify superoxide radicals (Broadley et al.,

2011; Cakmak, 2000). Expansins and nodulins were found to be downregulated in *Arabidopsis* (Van De Mortel et al., 2006). Although there are some Zn deficiency-caused gene expression changes in plants which are generally accepted to be Zn-regulated, a lot of adaptational reactions remain incomprehensible and obscure. In addition, most research concerning gene expression changes in plants due to Zn deficiency has been carried out in the model plant *Arabidopsis thaliana* as well as in yeast and also some research has been done in rice and spinach. To our knowledge, no investigation about differential gene expression between maize plants with sufficient or deficient Zn supply has been made.

Additionally, the three maize transcriptomes induced by N, P or Zn deficiency have not been compared before. Maize belongs to the most important crops worldwide and, as a nutrient-demanding plant, is affected strongly by N, P or Zn deficiency, which is why RNA-sequencing on fully nutrient-supplied maize as well as on deficient maize plants was performed to compare the proteomes. Thereby, it was possible to identify typical N, P and Zn deficiency response genes to be differentially expressed.

1.4 Function and heritability of epigenetic mechanisms in plants

Epigenetics are defined as heritable changes in gene activity without changing the DNA sequence (Weinhold, 2006). There are several epigenetic mechanisms (**Figure 1**) with known, but also unknown functions. Epigenetic modifications include methylation, acetylation, phosphorylation, ubiquitylation and sumoylation (Weinhold, 2006). Epigenetic mechanisms help plants increase their plasticity in adaptation to environmental changes, e.g. by increasing their methods of gene regulatory activities.

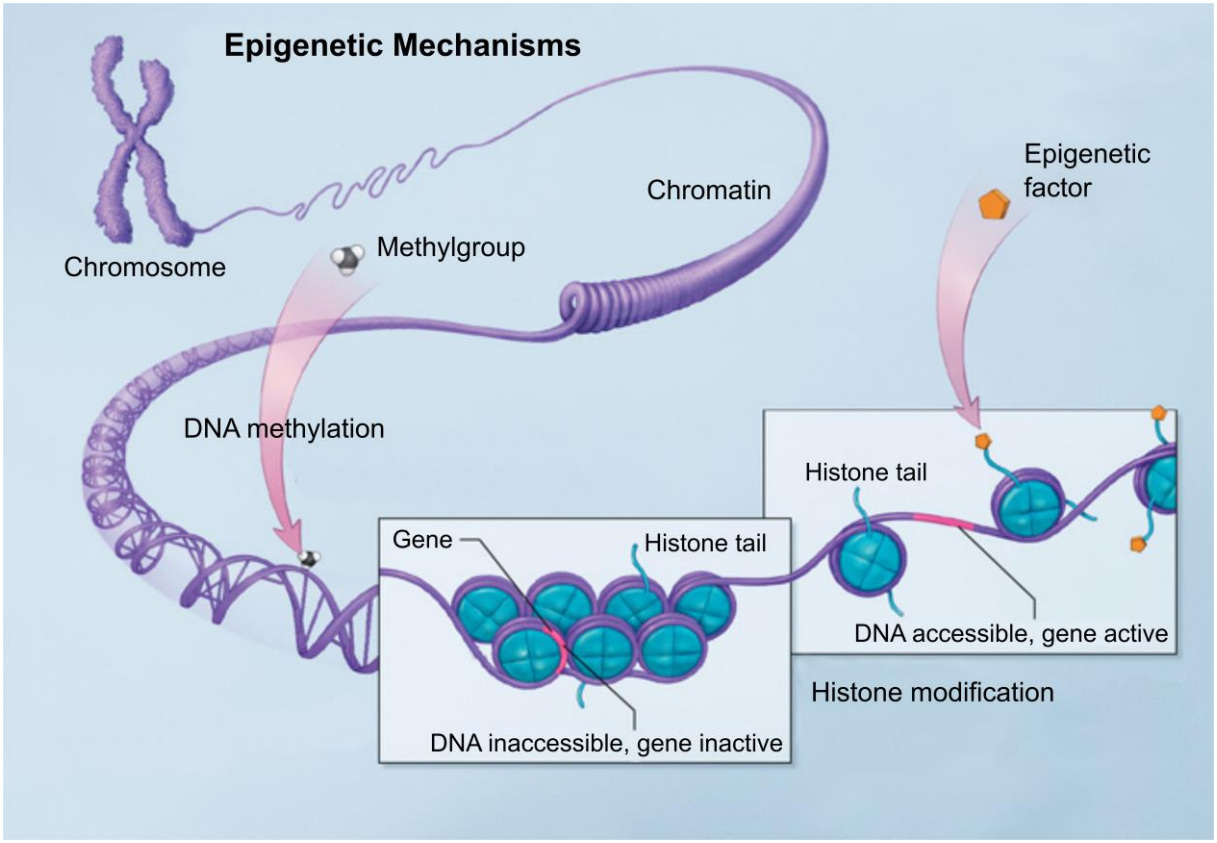

Figure 1: Overview about epigenetic mechanisms.
(Source: http://commonfund.nih.gov/epigenomics/figure.aspx)

DNA methylation and histone modifications were shown to participate in various plant functions, like pathogen response, genome stability, protection from DNA damage, preserving nucleotide sequences, heterosis, imprinting, paramutation and regulation of transposable elements and gene expression (Pikaard and Mittelsten Scheid, 2014; Putiri and Robertson, 2011; Reinders et al., 2009; Vidalis et al., 2016). It is known that epigenetic changes, like histone and DNA methylation, influence the packaging of chromatin, thereby producing eu- or heterochromatin. This creates easy or blocked access of the transcription machinery to the DNA, respectively, and thereby regulates gene expression (Bender, 2002; Chinnusamy and Zhu, 2009). Furthermore, epigenetic mechanisms regulate the frequency and chromosomal distribution of recombination (e.g. crossover) events and the movement of transposable elements (Mirouze et al., 2009, 2012; Mlura et al., 2001; Putiri and Robertson, 2011; Yelina et al., 2015).

DNA methylation and histone modifications are believed to influence each other. For example, cytosine methylation seems to induce histone modifications by engaging methyl-DNA binding proteins. These proteins then signal to histone-modifying enzymes and chromatin-remodeling factors. Subsequently, heterochromatin is formed with the help of these factors and thereby the access to the DNA obstructed (Bender, 2002).

Maize is an interesting plant for epigenetic research and a couple of gene regulatory epigenetic functions have been shown in maize including their heritability to following generations. Among these mechanisms are paramutation and genomic imprinting. In the phenomenon of paramutation, one allele of a gene transfers epigenetic information to another of the gene's alleles and thereby silences it. (Chandler, 2007; Haring et al., 2010; Pilu, 2015; Slotkin and Martienssen, 2007). The first allele is called paramutagenic and the second one paramutable. This expression change is heritable over generations and the resulting phenotype corresponds to the paramutagenic allele's expression, thereby overriding Mendel's laws. The former paramutable allele becomes paramutagenic on his part in the next generations and can in turn silence other alleles. A comparatively well investigated example of this phenomenon is the maize *b1* locus involved in the activation of the anthocyanin pigmentation pathway (Bender, 2002; Chandler, 2007; Haring et al., 2010; Pilu, 2015). The *b1* locus contains a highly expressed allele, the B-I allele and a very low expressed allele, the B' allele (**Figure 2**). Plants with a high expression of the *b1* locus produce purple anthocyanin pigments, giving the plants a darker purple color while maize with a low expression appears green. The DNA sequence of the two alleles is identical. In heterozygous plants, the paramutagenic B' allele causes a loss of activity by transferring epigenetic information (usually hypermethylation) to the B-I allele which subsequently also

becomes hypermethylated. The B-I allele thereby switches to a B' state and becomes paramutagenic as well. Offspring of crosses between B' and B-I are all green, indicating the heritable quality of the paramutation and the ability of the formerly paramutable allele to exert paramutation, too (Bender, 2002; Chandler, 2007; Haring et al., 2010; Pilu, 2015).

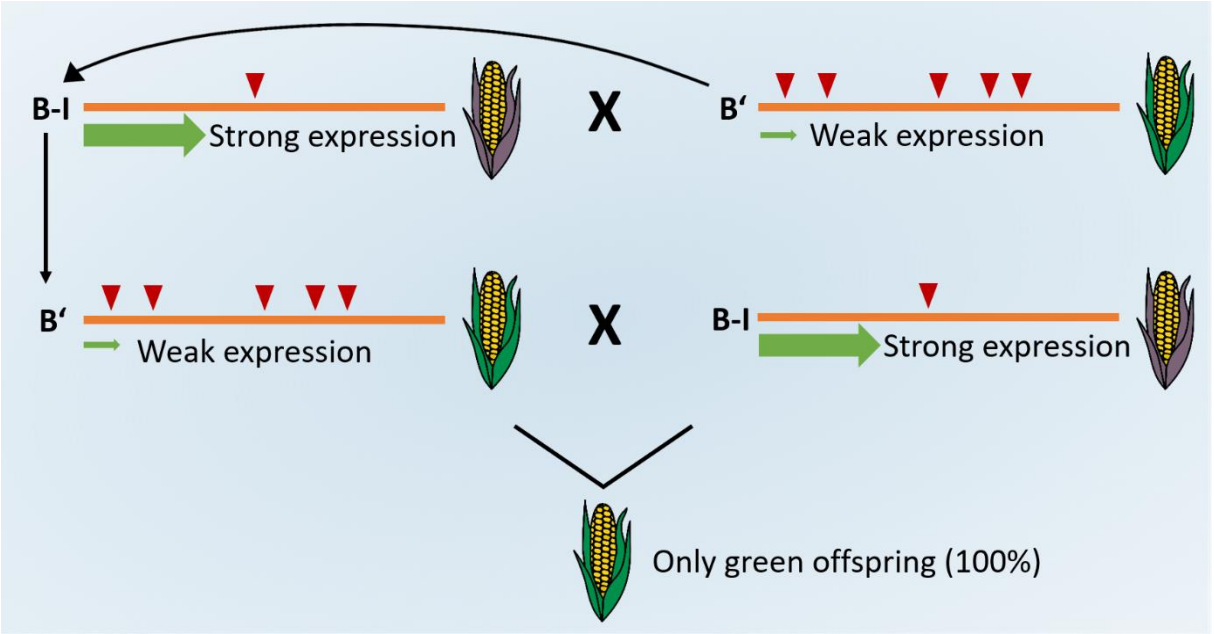

Figure 2: Paramutation of the maize *b1* locus.
The paramutable allele B-I is highly expressed (big green arrow) and produces purple colored maize plants. The B' allele which is weakly expressed (small green arrow), produces green plants and in heterozygous plants can transfer epigenetic information (red triangles) onto the B-I allele which in turn becomes B' as well and gains the paramutagenic function. Crossing this epigenetically changed (hypermethylated) allele with a B-I allele results in only green plants.

A no less interesting case of epigenetic gene expression regulation is genomic imprinting (**Figure 3**). Imprinting describes a mechanism in which only one of two alleles of a gene in a diploid organism is active while the other is suppressed, depending on the epigenetic status of the maternal and paternal alleles (Dickinson and Scholten, 2013; Jahnke and Scholten, 2009; Scholten, 2010; Slotkin and Martienssen, 2007). Usually, silencing one of the alleles during imprinting is associated with DNA methylation of this allele as already observed for example in maize, rice and

Arabidopsis (Dickinson and Scholten, 2013; Feil and Berger, 2007; Jahnke and Scholten, 2009; Luo et al., 2011; Scholten, 2010). It was believed that, while imprinting in mammals occurs in the embryo as well as in non-embryonic tissues, the phenomenon in plants was restricted to tissues other than the embryo. Anyways, in maize the maternally expressed in embryo 1 (*mee1*) gene was found to be imprinted in endosperm as well as in the embryo (Dickinson and Scholten, 2013; Jahnke and Scholten, 2009; Scholten, 2010). For this gene, only the maternally inherited allele was active and contained only a low amount of methylation while the paternal allele was inactive and highly methylated.

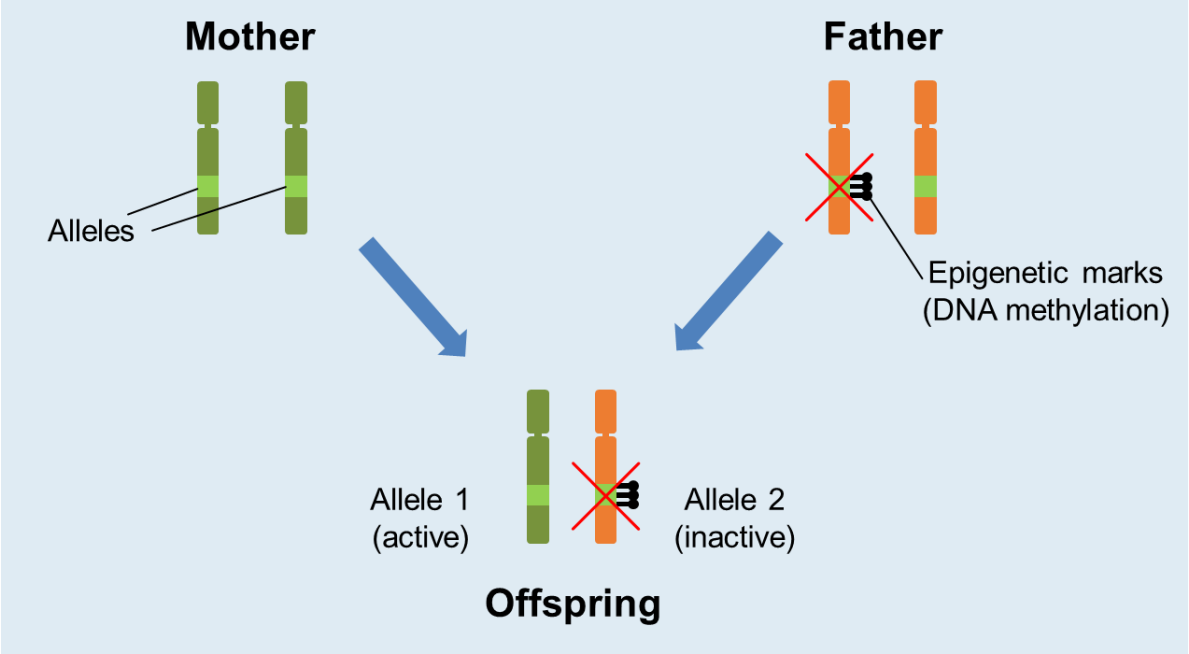

Figure 3: Schematic overview of genomic imprinting.
Usually both alleles of a gene inherited by the parents are expressed. Due to genomic imprinting, one of the alleles is inactivated by epigenetic factors, depending on whether it is paternally or maternally inherited. Here, the paternal allele is silenced by DNA methylation.

1.5 DNA methylation methods in plants

During DNA methylation a methyl group is placed on a cytosine base of the DNA strand (**Figure 4**).

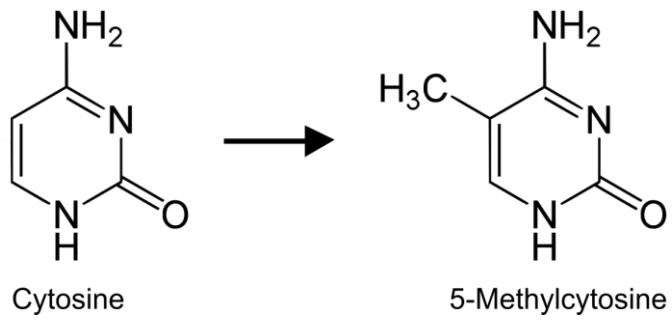

Figure 4: Cytosine and 5-Methylcytosine.

In plants, this occurs in all possible cytosine (C) contexts (Pikaard and Mittelsten Scheid, 2014; Secco et al., 2015; Yong-Villalobos et al., 2015), namely the symmetrical CG and CHG contexts, as well as the asymmetrical CHH context, with G being guanine and H being any base but guanine (**Figure 5**).

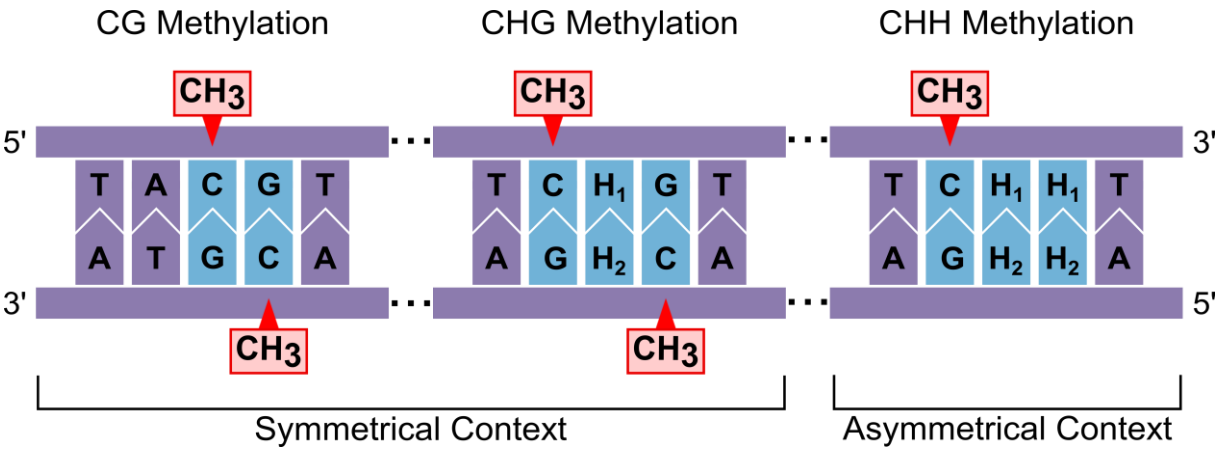

Figure 5: The three different cytosine methylation contexts occurring in plants. CG and CHG contexts belong to the symmetrical contexts while CHH is asymmetrical. H_1 = any base but guanine, H_2 = any base but cytosine.

Two major methodically different categories for DNA methylation can be differentiated: maintenance and *de novo* methylation. Maintenance methylation is a mechanism by

which during cell replication the existing methylation positions are directly copied from the parent strand and established in the newly synthesized DNA strand in exactly the same pattern (**Figure 6**).

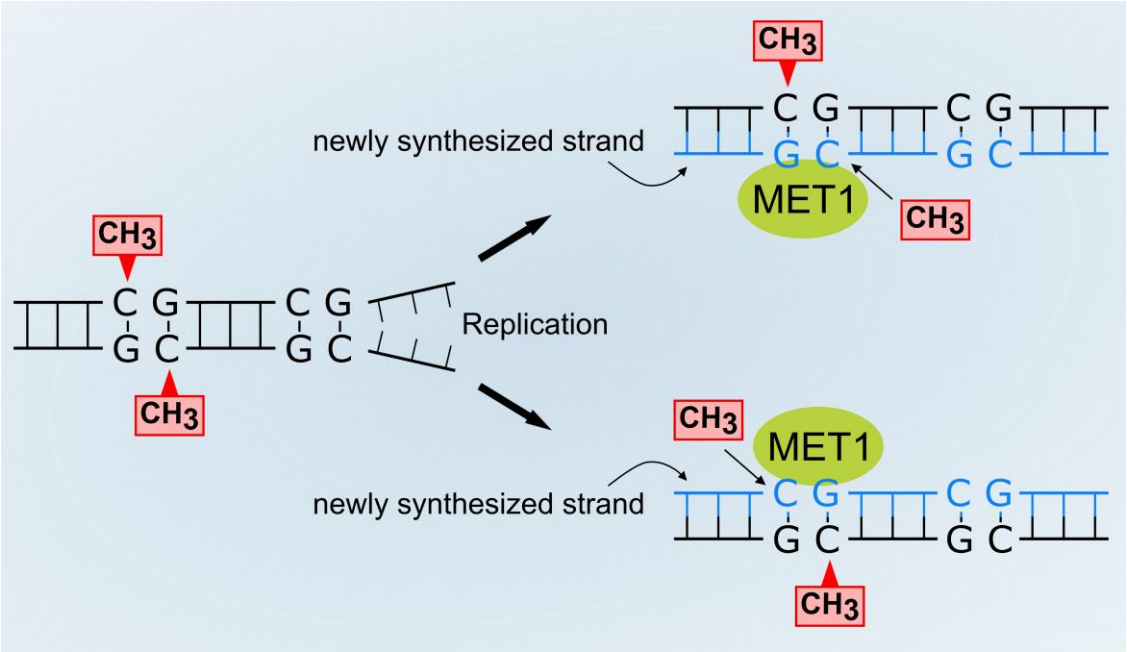

Figure 6: Maintenance methylation with MET1.
During maintenance methylation, the methylation information from the template strand is copied to the newly synthesized strand.

This is methodically straightforward in the symmetrical CG and CHG contexts and is accomplished by the maintenance enzyme Methyltransferase1 (MET1) in the CG context and chromomethylase3 (CMT3) in the CHG context (Eichten et al., 2014; Pikaard and Mittelsten Scheid, 2014; Secco et al., 2015).

As the CHH context does not provide the methylation information on the template strand during replication, CHH motifs require *de novo* methylation via RNA-directed DNA methylation (RdDM) after replication (**Figure 7**). Though RdDM occurs in all contexts to pose *de novo* methylation on cytosines, it is most prominent in the CHH context. As soon as methylations in the symmetrical contexts are established, they can be maintained via maintenance methylation, rendering them less dependent on RdDM

during subsequent rounds of DNA replication (Matzke and Mosher, 2014). In the *de novo* RdDM pathway RNA polymerase IV produces single-stranded RNA transcripts that are subsequently converted to double-stranded RNAs by RNA-dependent RNA polymerase 2 (RDR2) and then processed to 24-nucleotide small interfering RNAs (siRNAs) by Dicers. These are loaded onto Argonaute 4 (AGO4) and guided to RNA polymerase V-transcribed RNA scaffolds. Finally, Domains 10 Rearranged Methyltransferase 2 (DRM2) is recruited to place *de novo* methylations on the DNA (Dowen et al., 2012; Lister et al., 2009; Matzke and Mosher, 2014; Secco et al., 2015).

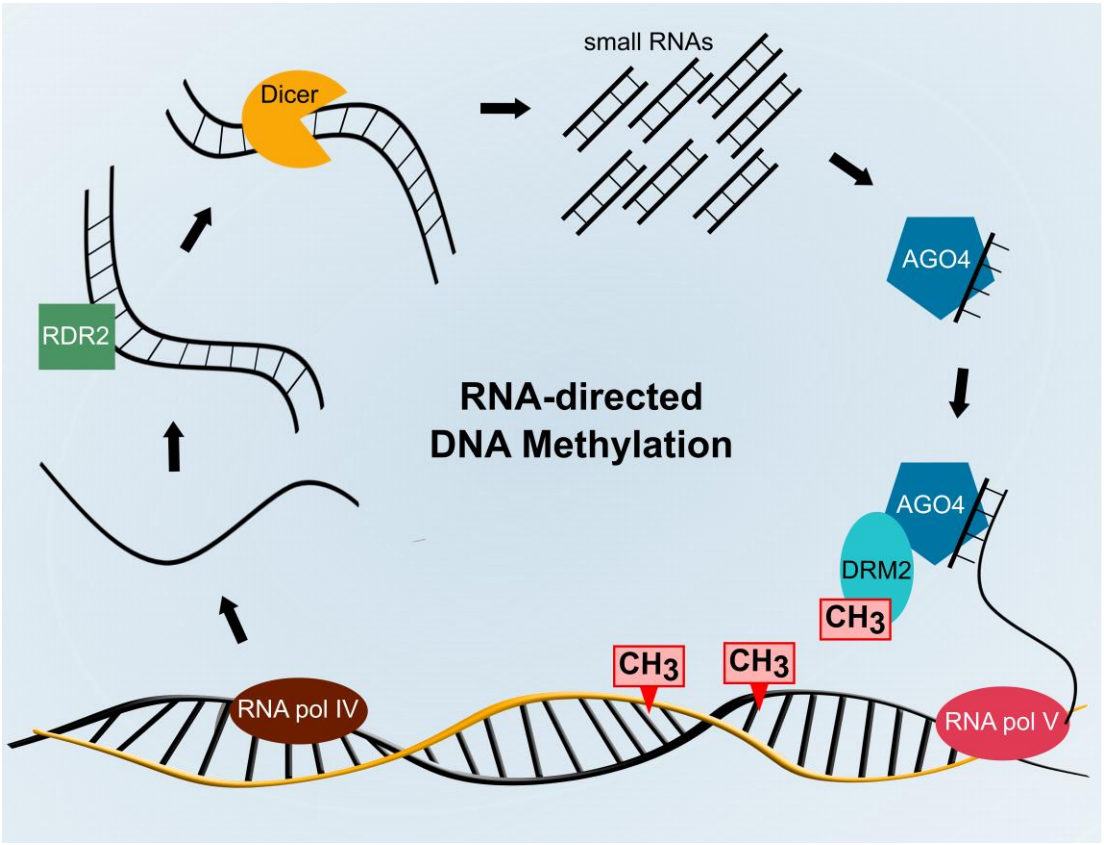

Figure 7: Overview about the steps during RNA-directed DNA methylation (RdDM).

In addition to mechanisms adding methylation to the DNA, there are also ways for the plant of removing them. This can happen passively, when there is a lack of maintenance methylation during replication or DNA repair. Furthermore, active loss of 5-methylcytosine happens through Repressor of silencing 1 (ROS1) and Demeter

(DME) proteins. These contain DNA glycosylase domains for base excision repair. The fact that methylation can be actively set and removed by plants suggests that dynamic regulation of DNA methylation is critical for the plant and influenced by environmental conditions (Eichten et al., 2014; Park et al., 2017; Pikaard and Mittelsten Scheid, 2014).

1.6 DNA methylation adaptation

How plants adapt to environmental changes via DNA methylation and how this influences plant internal processes like gene expression is a much debated and researched topic. There are some assumptions generally accepted, but nonetheless many research results are quite contradictory. Not only do these contradictions occur among different plant species but also within one species. Often, a hypermethylation of transposable elements (TEs) is associated with blocking of the TEs while increasing gene expression of nearby genes (Secco et al., 2015), but hypermethylated TEs were also already found near downregulated genes (Ahmed et al., 2011; Eichten et al., 2012). A high DNA methylation in genes or near transcription start sites (TSS) is often believed to shut down expression but there are also examples where methylated genes are moderately expressed and even cases where gene body methylation seems to stabilize expression (Li et al., 2015; Suzuki and Bird, 2008). Equally inconsistent are the findings about DNA methylation change due to nutrient deficiencies. An interesting example are two studies in which DNA methylation adaptation to phosphorus deficiency in *Arabidopsis thaliana* was investigated and in which one team found considerable changes in DNA methylation and the other team only very minor DNA modifications (Secco et al., 2015; Yong-Villalobos et al., 2015). These contradictory findings show that there is still a lot to be learned about the functions of DNA methylation, its adaptation to nutrient stresses and its correlation to gene expression.

1.7 Objective of this research project

Although there has been some research concerned with investigation of the adaptation of DNA methylation to environmental stresses and thereby also to nutrient stresses, most of them are in *Arabidopsis* and only a small number in maize. As most crop plants, for example cereal plants, are very different from *Arabidopsis*, composing a totally different plant family, definitely not all functions in *Arabidopsis* can be translated par for par on crop plants. One big difference between *Zea mays* and *Arabidopsis thaliana* is the size of the genome. Maize has a genome size of about 2.3 gigabases, which is about 18 times bigger than the genome of *Arabidopsis thaliana* which only comprises 125 megabases. Additionally, maize is composed of about 85% transposable elements, whereas the *Arabidopsis thaliana* genome only contains about 10% TEs (Arabidopsis Genome Initiative, 2000; Feschotte et al., 2002; Schnable et al., 2009; Tenaillon et al., 2011; Zhang and Wessler, 2004). Therefore, nutrient deficiencies were applied, namely nitrogen, phosphorus and zinc, to *Zea mays* to contribute to the understanding of DNA methylation adaptation in maize and additionally the influence of three very important plant nutrients on methylation was compared to investigate if DNA methylation adapts in a nutrient-specific way, rather than adapting as a general stress response.

DNA methylation was investigated in the maize inbred line B73. Due to the large genome of maize, which makes a deep coverage of the DNA methylation pattern via whole genome sequencing expensive and less efficient, our method of choice was reduced representation bisulfite sequencing (RRBS) under usage of the restriction enzyme MspI (Li et al., 2014). By cutting the DNA with a restriction enzyme specific for a CG-containing motif and selecting for small DNA fragments, the sequencing libraries

became enriched for CG-rich regions (Martinez-Arguelles et al., 2014; Smith et al., 2009). As a result, a representative high coverage methylation profile was achieved.

Research concerning influence of DNA methylation on gene expression produced many different outcomes, ranging from nearly complete lack of correlation in *Brachypodium distachyon* (Roessler et al., 2016) over solid correlations found in *Arabidopsis thaliana* (Yong-Villalobos et al., 2015). Additionally, even where relatively strong correlation between methylation and gene expression was found, a clear pattern about whether hypo- or hypermethylation cause up- or downregulation of gene expression, could not be established (Yong-Villalobos et al., 2015).

Therefore, this work aims at addressing these questions by investigating methylation changes in maize plants and corresponding gene expression changes. Thus, RNA-seq was applied to the same maize root samples that were used for RRBS.

Summarizing, the research described here is supposed to contribute to the understanding of dynamic DNA methylation adaptations due to different nutrient stresses in an important crop plant and to investigate possible functions of these changes, as for example gene expression regulation. Understanding DNA methylation dynamics and their functions might provide useful new possibilities for plant breeding and in crop protection.

2 Materials

2.1 Maize nutrient solution for growth of B73 plants

Basic full nutrient solution for maize was used to grow control plants in hydroponic culture.

Table 1: Basic maize nutrient solution for sufficient supply.

Nutrients for Ctrl samples	**Concentration**
K_2SO_4	0.5mM
$MgCl_2$	0.6mM
$Ca(NO_3)_2$	2.5mM
KH_2PO_4	0.1mM*
H_3BO_3	1µM
$MnSO_4$	0.5µM
$ZnSO_4$	0.5µM
$CuSO_4$	0.2µM
$(NH_4)_6Mo_7O_{24}$	0.01µM
Fe-Sequestrene	100µM**
Modifications for -N samples	**Concentration**
$Ca(NO_3)_2$	90µM
Modifications for -P samples	**Concentration**
KH_2PO_4	18µM
Modifications for -Zn samples	**Concentration**
Fe-EDTA	300µM
$ZnSO_4$	0.1µM (for 24h in week 3 and 4)

* Raised to 0.2mM in the third week and to 0.5mM in the fourth week for Ctrl samples.

** Raised to 200µM at first nutrient solution change and to 300µM at the second solution change for Ctrl, -N and -P samples. Not present in -Zn samples.

2.2 Kits

Table 2: Kits used for DNA, RNA and small RNA extraction and/or quantification.

Kit	Purpose	Merchant
DNeasy Plant Mini Kit	DNA extraction from maize root samples	Qiagen
innuPREP Plant RNA Kit	RNA extraction from maize root samples	analytikjena
innuPREP Micro RNA Kit	Small RNA extraction from maize root samples	analytikjena
Small RNA Kit	Quantification of small RNAs from maize root samples on the Agilent 2100 Bioanalyzer	Agilent Technologies

2.3 Instrumental equipment

Table 3: Instrumental equipment used.

Instrument	Purpose	Merchant
2100 Bioanalyzer	Quantification and quality check of RNA and small RNAs from maize root samples	Agilent Technologies
Nanodrop 2000c Spectrophotometer	Quantification and quality check of RNA and DNA from maize root samples	Thermo Fisher Scientific
Qubit Fluorometric Quantitation	Quantification and quality check of DNA from maize root samples	Thermo Fisher Scientific
UV-Vis Spectrophotometer U-3300	Measurement of phosphorus content in maize leaf samples	Hitachi, Schwäbisch Gmünd, Germany
EuroVector Euro EA 3000 Elemental Analyzer	Measurement of nitrogen content in maize leaf samples	HEKAtech GmbH
iCE 3000 Series Atomic Absorption Spectrometer	Measurement of zinc content in maize leaf samples	Thermo Fisher Scientific
Illumina Hiseq 2000	Sequencing of RRBS library	Illumina (provided by Beijing Genomics Institute, HongKong, China)
Illumina Hiseq 4000	Sequencing of RNA samples	Illumina (provided by Beijing Genomics Institute, Hongkong, China)

2.4 Bioinformatic applications

Table 4: Bioinformatic tools used for methylome and transcriptome analyses.

Application	Purpose	Reference
FastQC	Quality check of clean data (RNA-seq, RRBS)	Babraham Bioinformatics
FastX-Toolkit	Cut off the first 4 and last 6 bp of the RRBS reads	Hannon Lab
BS-Seeker2	Mapping of RRBS reads to *Zea mays* reference genome	Guo et al., 2013
Bowtie 2	Short read mapper during alignment	Langmead and Salzberg, 2013
DMRcaller	Determination of DMRs	Zabet and Tsang, 2015
HISAT2	Alignment of RNA-seq reads to *Zea mays* reference genome	Kim et al., 2015
Cufflinks suite of tools	Assembly of RNA-seq reads, merging of assemblies, determination of DEGs	Trapnell et al., 2010
BEDTools	Determination of closest gene to each TE	Quinlan and Hall, 2010

2.5 Plant material

Seeds of *Zea mays* B73 inbred line were provided by Professor Albrecht E. Melchinger of the Department of Plant Breeding, University of Hohenheim, Stuttgart.

2.6 *Zea mays* reference databases

Table 5: *Zea mays* reference genome, annotation data and list of transposable elements used in the analyses.

Database	Purpose	Reference
Zea mays reference genome (AGPv3)	Alignment of RRBS and RNA-seq reads	Sen et al., 2009
Maize annotation files (AGPv3)	Transcript assembly in RNA-seq analysis and determination of DMRs in genes/promoters	Sen et al., 2009
Transposable elements (ZmB73v3)	Determination of DMRs in TEs and correlation of TE methylation with gene expression	Unité de Recherche Génomique Info (Jamilloux et al., 2017)

2.7 Services

Restriction digest of DNA from maize root samples, bisulfite treatment and library preparation as well as sequencing for methylome analysis was done by Beijing Genomics Institute (BGI), HongKong, China. For the transcriptome analysis the library preparation and sequencing were also done by BGI.

3 Methods

3.1 Plant growth conditions

Growth of the maize plants was done in a hydroponic system under controlled conditions in a climate chamber with simulated day length of 16 h at 25 °C and 8 h night length at 20 °C. Humidity amounted to 60–80% and photosynthetically active photon flux density (PFD) was 400 µmol m^{-2} s^{-1}. First, seeds from the maize B73 inbred line were surface-sterilized by rinsing them for 2 minutes in a 10% H_2O_2 solution. The solution was afterwards washed away under distilled water. The seeds stayed in a 10mM $CaSO_4$ solution for 24 hours and were then laid between foam sheets and filter paper soaked in a 3mM $CaSO_4$ solution for 4 days to germinate and develop first roots. During the first three days they were kept in dark until germination could be seen. Afterwards, when the roots were >3cm long, the seedlings were put for 3 days into pots (6 plants each) containing 2.8l of a diluted maize nutrient solution containing 1/5th of all nutrients of the basic solution (**Table 1**). After 3 days, the seedlings were exposed to different treatments in 2.8l pots, each pot now containing only 2 plants (**Figure 8**). For control conditions, full maize nutrient solution was used with KH_2PO_4 being raised to 0.2mM in the third week and to 0.5mM in the fourth week after starting the treatments. Nitrogen deficiency samples only got 90µM of $Ca(NO_3)_2$ to induce deficiency. For phosphorus deficiency, the amount of KH_2PO_4 was reduced to 18µM. Otherwise, both -N and -P plants were treated the same as the control, except that they got 0.3mM KH_2PO_4 when control plants got 0.5mM. For Zn deficiency plants the amount of KH_2PO_4 was not raised but kept at 0.1mM. Instead of Fe-Sequestrene, 300µM of Fe-EDTA were added to the solution in case Fe-Sequestrene contained traces of Zn. Zn was not added to the solution at all, but in week 3 and 4 of the

treatment, 0.1µM $ZnSO_4$ were given to the solution for 24h to prevent dying of the plants. For all pots, the first nutrient solution change was done after 7 days and from then on, every 3 days till the harvest. 5 weeks after germination (4 weeks after treatment start) the plants were harvested.

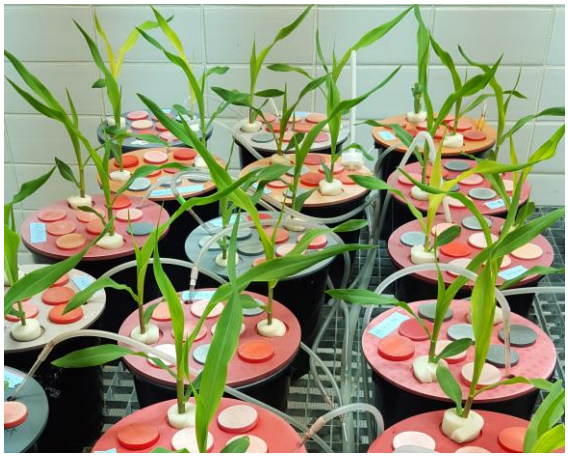

Figure 8: Plant growth.
Hydroponic growth of maize plants with different treatments.

3.2 Nutrient analysis

For the nutrient analysis, the second and third youngest leaves were taken from each plant. The two plants from one pot were pooled, so that for the control and each treatment 3 replicates with two plants per replicate were used. The leaf material was measured for nitrogen, phosphorus and zinc content. The samples were ground to a fine powder before being digested via microwave (VDLUFA, 2011). Phosphorus content was subsequently measured via UV-VIS spectroscopy while nitrogen and zinc content were measured after Kjeldahl (Kjeldahl, 1883).

3.3 Reduced representation bisulfite sequencing of DNA samples and methylome analysis

For the methylome analysis root material was harvested. Here again, material of the two plants from one pot were pooled resulting in three replicates with two plants per replicate for each sample. After harvest of maize roots, the material was ground to a fine powder and DNA was extracted via Qiagen DNeasy Plant Mini Kit according to the manual. If necessary, the DNA was concentrated and cleaned via alcohol precipitation. DNA samples were checked for quantity and quality via Thermo Scientific Nanodrop 2000c Spectrophotometer and Qubit Fluorometric Quantitation. Only samples with an OD260/280 bigger or equal to 1.8 were used for further processing. Reduced representation bisulfite sequencing (RRBS) was used (**Figure 9**) to monitor methylation (Li et al., 2014), in which a partial high-density coverage of the genome methylation profile allowed a representative genomic view (Martinez-Arguelles et al., 2014; Smith et al., 2009). The samples were send to Beijing Genomics Institute (BGI, China) where the DNA was digested with MspI and fragments of length 40 to 220bp were selected. The DNA was treated with bisulfite followed by 100bp paired-end library construction and sequencing on Illumina Hiseq 2000.

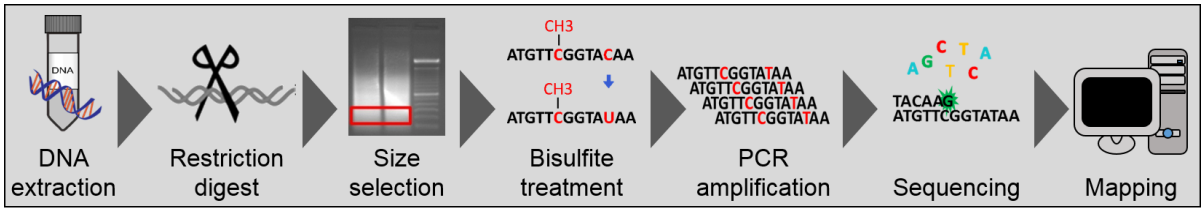

Figure 9: The steps of reduced representation bisulfite sequencing.

A quality check on the clean data provided by BGI was done with FastQC (Babraham Bioinformatics). To increase the quality further, FastX-Toolkit by Hannon Lab was used to cut off the first 4 and last 6 bp of all reads. Mapping was done via BS-Seeker2 (Guo

et al., 2013) under usage of the *Zea mays* reference genome (AGPv3), which was provided by the Maize Genetics and Genomics Database (Sen et al., 2009). BS-Seeker2 virtually cut this genome with MspI and size-selected sequences of 20 to 400bp length. Using this reduced representation reference genome increased mappability rate and alignment accuracy. A broader range (20 to 400bp) of size-selection was used for the virtual reduced representation genome than for the digested DNA samples to account for inaccuracies during size-selection of the digested DNA samples which might contain smaller or longer sequences than the intended 40-220bp length. During alignment, default settings were used with bowtie2 as short read mapper (Langmead and Salzberg, 2013). Default settings were also used to call methylation levels after mapping. DMRcaller (Zabet and Tsang, 2015) was then used to determine differentially methylated regions (DMRs) by pooling the methylation level information from all three replicates of one sample. Smoothing was done via noise_filter with triangular kernel (Hebestreit et al., 2013) for computing differentially methylated cytosines and the score test, as specified by DMRcaller, for determination of DMRs. DMRs were characterized as being 50-500bp long, containing at least 4 cytosines, having a methylation difference of at least 40% in comparison to the control and a p-value of ≤ 0.01. DMRs located within genes were determined with gene information from maize annotation files (AGPv3) provided by the Maize Genetics and Genomics Database (maizeGDB) (Sen et al., 2009). To find out which DMRs are located in promoter regions, the region comprising 2000bp upstream of a gene was defined as promoter region. For identification of differentially methylated transposable elements a list of transposons provided by Unité de Recherche Génomique Info (Jamilloux et al., 2017) was used.

3.4 RNA Sequencing on total RNA samples and proteome analysis

For transcriptome analysis via RNA sequencing (**Figure 10**) total RNA was extracted from root material (the same material as was used for DNA extraction) via analytikjena innuPREP Plant RNA Kit according to the manufacturer's manual. The quantity and quality of the RNA were determined via measurement in the Thermo Scientific Nanodrop 2000c Spectrophotometer as well as in the Agilent 2100 Bioanalyzer. Like the DNA samples, the RNA samples had to have a purity of OD260/280 ≥ 1.8 to be used for further processing.

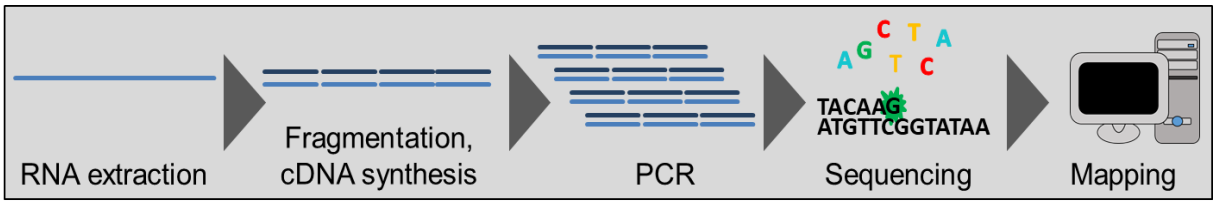

Figure 10: The steps of RNA sequencing.

Quality check via the Bioanalyzer as well as Truseq 160bp short-insert library construction and 100bp paired-end sequencing on Illumina Hiseq 4000 was done by Beijing Genomics Institute (BGI) in China. The clean data received from BGI was quality checked via the FastQC tool (by Babraham Bioinformatics). The reads were then aligned via HISAT2 (Kim et al., 2015) to the *Zea mays* reference genome (AGPv3), which is provided by the maizeGDB (Sen et al., 2009), with default options except for adding the options --phred64, --dta-cufflinks, --no-mixed and --no-discordant. As annotations for aiding in transcript assembly maize annotation files (AGPv3) were used, which were also provided by the maizeGDB (Sen et al., 2009). The assembly was done with cufflinks from the cufflinks suite of tools (Trapnell et al., 2010) with default options and the --GTF-guide and --no-effective-length-correction. After merging the assemblies with cuffmerge (with -g and -s options), cuffdiff was used

to find differentially expressed genes with --compatible-hits-norm, -b, -u and otherwise default options.

3.5 Correlating transcriptome data with methylation information

To determine if DMRs in gene body or promoter region influence gene expression of that gene, Fisher's exact test on a 2x2 contingency table was applied. A 5% significance level was used. The contingency table contained the number of genes that were both differentially methylated and differentially expressed, the number of genes only differentially methylated, the number of genes only differentially expressed and the number of genes neither differentially expressed nor methylated. However, only genes were taken into account for the test which were covered by the reduced representation genome (RRG) with at least 500 bases. This cutoff was set to avoid comparing a lot of genes for which no methylation information but only expression information was available. This reduced false negative results (the incidence that a gene for which no methylation information was present was stated to contain no DMR). A minimum of 500 covered bases was chosen as a compromise between losing too many genes with DMRs and keeping too many genes without methylation information. For investigation of whether differential methylation in transposable elements influences the expression of the closest gene, first BEDTools was used to determine the closest gene for each TE regardless of being upstream or downstream of the TE (Quinlan and Hall, 2010). Only those transposable elements were taken into account, which were covered by the reduced representation genome at all. This difference in setting the cutoff for genes and TEs was used because many TEs are very short und setting another cutoff lost a lot of TEs. Again, via Fisher's exact test with a significance level of 5% on a 2x2 contingency table, the dependence of differential methylation in

TEs and differential expression of the closest gene was determined. For determination of linear correlation between differentially methylated genome features and gene expression, scatter plots were done with methylation proportion difference against the log_2 fold change of expression.

3.6 Quantification of small RNAs

For investigation if a lower level of methylation is associated with a lower amount of small RNAs (because they are involved in *de novo* methylation), small RNA was extracted from the same root material which was used for total RNA and DNA extractions. The extraction was done according to the analytikjena innuPREP Micro RNA Kit Manual. The samples were tested in the Thermo Scientific Nanodrop 2000c Spectrophotometer for purity and only samples with OD260/280 ≥ 2.0 were used. The amount of small RNAs was determined in the Agilent 2100 Bioanalyzer according to the Agilent Small RNA Kit Guide by Agilent Technologies. The ratio between the amount of small RNAs (15-30nt length) and the total amount of RNA was determined. As 21-24 nt small RNAs are involved in the RdDM pathway, a 15-30nt small RNA range was chosen for measurement of the ratio to make sure that all 21-24nt small RNAs were taken into account. One-way ANOVA was used to test for significant changes in the amount of small RNAs.

4 Results

4.1 Plant phenotypes and nutrient content

Control plants of the maize B73 inbred line grown in hydroponic culture showed vigorous growth, while deficiency plants showed typical deficiency symptoms (**Figure 11A**): For nitrogen deficiency, restricted shoot growth resulting in an increased root-to-shoot biomass ratio (Boussadia et al., 2010), pale green leaf color due to decreased photosynthesis and chlorosis in older leaves were observed (Comadira et al., 2015). Likewise, in -P plants, reduced shoot and more complex root growth, resulting in increased root-to-shoot biomass ratio and dark green leaves with anthocyanin accumulation, especially in the stems, were indicative of typical phosphorus deficiency. The phenotypes of the -Zn plants indicated that the plants were highly stressed and showed typical Zn deficiency-induced symptoms with strongly stunted growth and small leave size as well as chlorotic marks on leaves (Cakmak, 2000; Hajiboland and Amirazad, 2010). **Figure 11B** shows the nutrient contents of the control and treatment plants. The line indicates the sufficiency threshold below which maize plants are usually not adequately supplied with a nutrient any more. For N, this threshold is at 3%, for P at 0.25% and for Zn at 20ppm (Camberato and Maloney, 2012; Sahrawat, 2014). The amount of each of the nutrients for control plants lied clearly above the sufficiency threshold. -N samples showed a sufficient amount for P with 0.50%, while N content was deficient with 1.79%. Zn content for -N samples was also slightly below the sufficiency threshold. This marginally too low Zn amount did probably not really affect the plants. They were much smaller than the control due to the lack of N so that they most likely did not need as much Zn as a healthy, well supplied growing maize plant. -P plants contained only insufficient amounts of P with 0.11% but sufficient

N and Zn amounts. -Zn plants had sufficient amounts of both N and P and a deficient amount of Zn with only 6.91ppm. The nutrient contents of the samples confirmed the specific, severe systemic nutrient deficiencies due to the treatments.

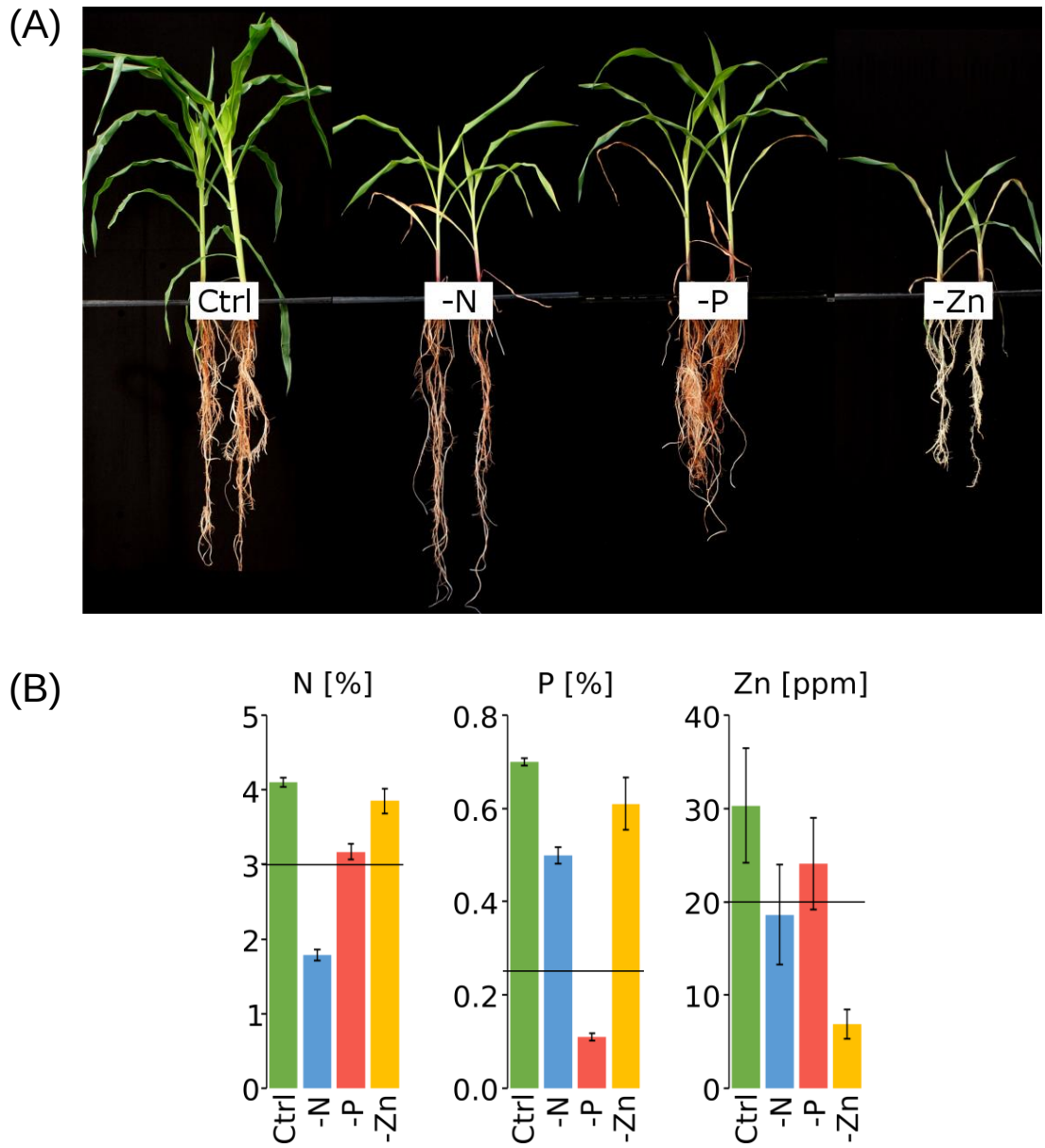

Figure 11: Plant phenotypes and nutrient content.
(A) Phenotypes of the maize plants. **(B)** Nutrient content of the maize plants. The black line indicates the minimum amount needed by maize. Error bars = standard deviation.

4.2 Evaluation of reduced representation bisulfite sequencing and nutrient deficiency-adapted methylomes

To get a cost-efficient and feasible as well as representative overview about methylation levels in the control and treatment samples, reduced representation bisulfite sequencing was applied which provided methylation information for about 14% of the maize genome in each sample with good coverage. The amount of the genome theoretically covered by the reduced representation genome was determined by virtually digesting the reference genome of maize with MspI and subsequent size-selection of DNA fragments of 40-220bp length. This revealed that about 14% of the real genome was covered by the reduced representation genome and about 18% of all cytosines. The method was applied in triplicate to the different treatment samples, each replicate containing root material from two plants. Bisulfite conversion rate after bisulfite treatment for each sample was >98% (between 98.41% and 99.18%). For all samples, independent of the cytosine context, about 90% of all cytosines were covered by at least 5 reads (**Figure 12**).

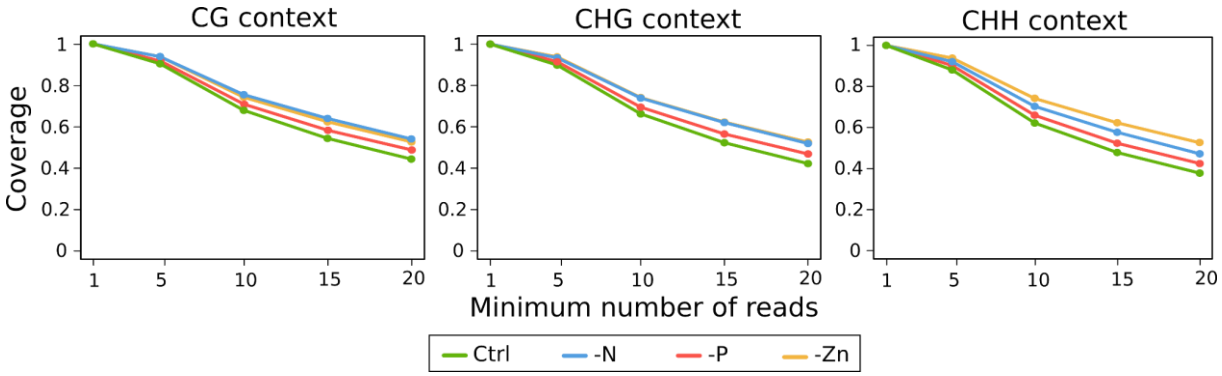

Figure 12: Cytosine coverage in CG, CHG and CHH context.

Mappability for all samples was > 48% (**Table 6**). Overall, RRBS processing showed sufficiently high coverage and mappability for reliable downstream analyses.

Table 6: Alignment output of RRBS libraries.
Values are averaged among replicates and shown in millions.

	Control	-N	-P	-Zn
Raw BS-read pairs	35.40	37.00	35.90	42.86
Multiple hits reads	0.13	0.13	0.13	0.13
Unmapped read pairs	18.20	19.00	18.38	21.78
Uniquely aligned read pairs	17.23	18.02	17.48	21.08
Mappability	**48.62%**	**48.70%**	**48.80%**	**49.16%**

A principal component analysis was performed on the methylation level of the cytosines of treatment samples -N, -P and -Zn (**Figure 13**). The 3D illustration shows the variance between the samples and each sample's replicates and that the replicates of the three samples do not overlap (**Figure 13A**). In the 2D picture it can be seen that the variance in principal component 1 (PC1) is mainly due to the differences between the samples though this variance explains only about 15% of the total variance (**Figure 13B**). PC2 mainly originates due to variance between the replicates of -P.

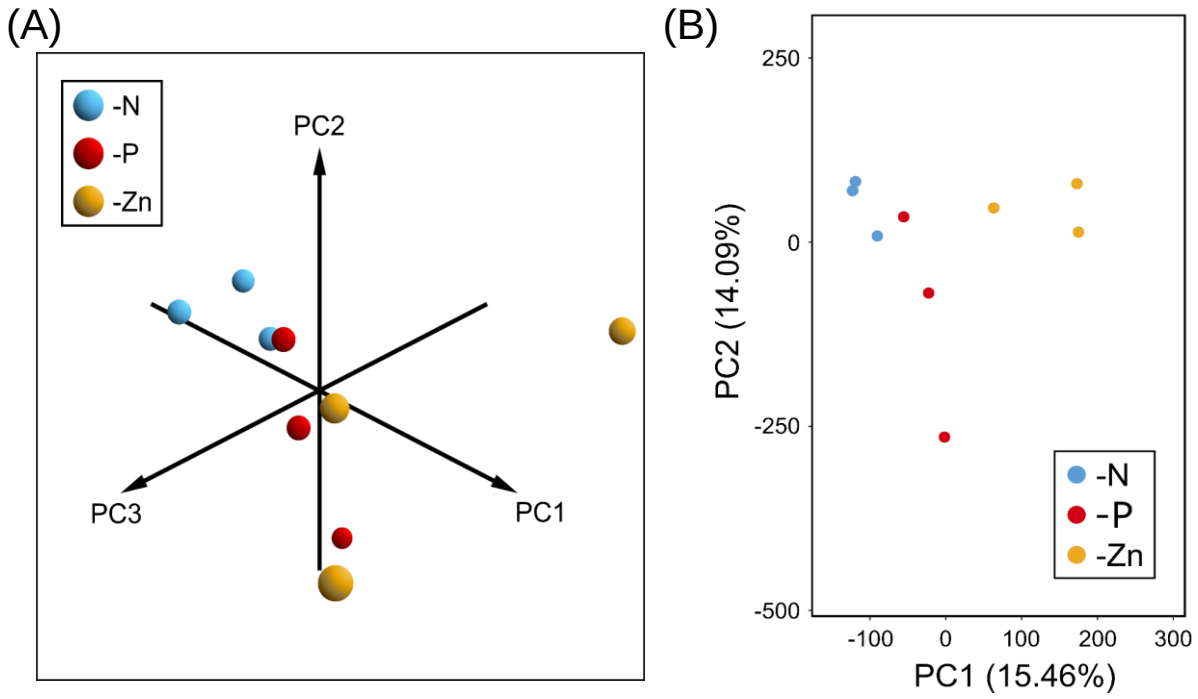

Figure 13: Principal component analysis (PCA) of cytosine methylation data.
PCA was conducted on methylation values of the cytosines determined by RRBS. **(A)** 3D illustration of PC1, 2 and 3. **(B)** 2D illustration of PC1 and 2.

Across the reduced representation genome, a massive loss of methylation in CG and CHG contexts for -N and -Zn was measured (**Figure 14A**). In the control, 26.6% of all cytosines in the CG context were methylated, whereas in -Zn samples only about half as many CGs were methylated and in -N samples even slightly less methylated CGs than in -Zn were found (**Figure 14A**). There was also a significant loss of methylation for -N samples in the CHG context, where 8.55% were methylated compared to 18.70% in control samples. A strong loss was also encountered for -Zn samples. Minor overall methylation loss for CG and CHG contexts in -P was also observed, with methylation levels of 22.48% in CG context and 16.15% in CHG context. However, the loss was much less pronounced than in -N and -Zn. The CHH context was almost unaffected by the deficiencies. Cytosines in this context showed only very low methylation. The methylation level was slightly further reduced in -N and -Zn samples, from 1.26% in control to 1.06% in -Zn and 0.96% in -N, while methylation in -P was even minimally larger (1.29%) than under control conditions.

The methylation distribution across whole chromosomes was analyzed by low-resolution profiles of the methylation levels via DMRcaller (**Figure 14B**). As a representative, the methylation of chromosome 1 is shown for the three different cytosine contexts and all samples. Especially in the CG and CHG contexts, the profiles show that the methylation was higher at the centromeric region and decreased towards the ends of the chromosomes. Again, the CHH context formed an exception, as here the higher methylation at the centromere was almost not visible. A rather uniform reduction of CG and CHG methylation was measured, but in -N and -Zn the reduction was a bit stronger in the centromeric region than towards the edges. In the CHH context, by contrast, -P was almost exactly the same as the control and the methylation

reduction in -N and -Zn was less pronounced in each chromosome than in the other two contexts.

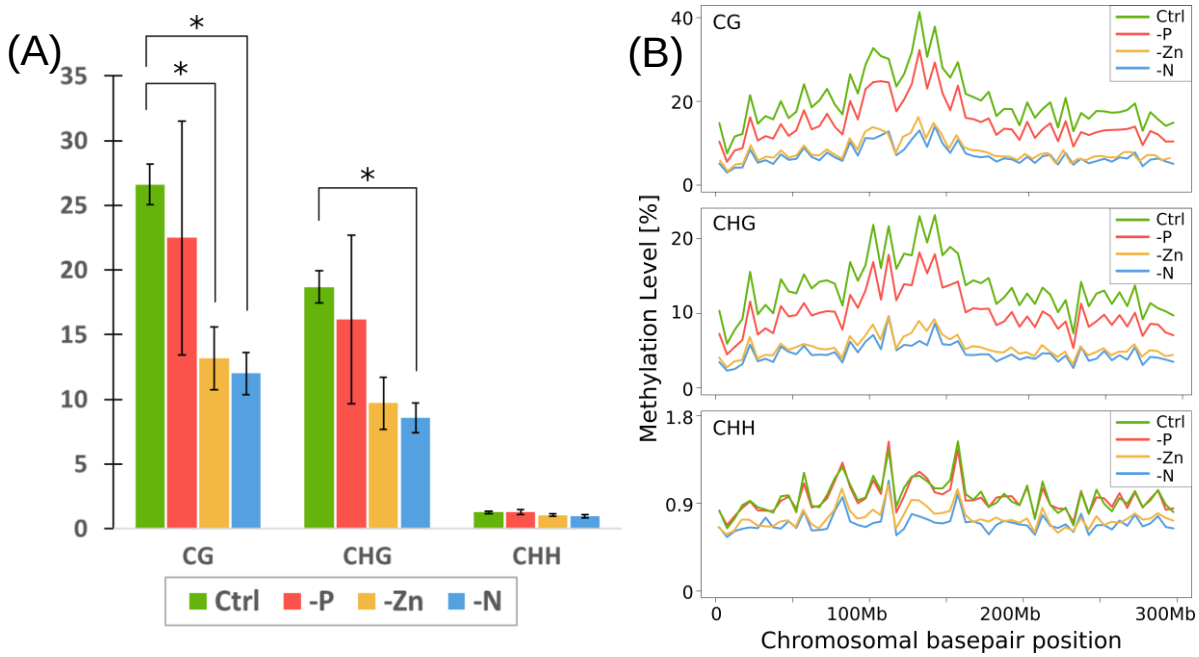

Figure 14: Overall methylation level in each context and sample.
(A) Methylation level averaged among the whole reduced representation genome. Error bars = standard deviation. **(B)** Average methylation level across chromosome 1 in a low-resolution profile in a grid of 5 million bases.

Even though a lot of methylation was lost in the deficiency samples, the relative contribution of each context to the total number of methylated cytosines was, however, more or less unaffected by the deficiencies (**Figure 15**).

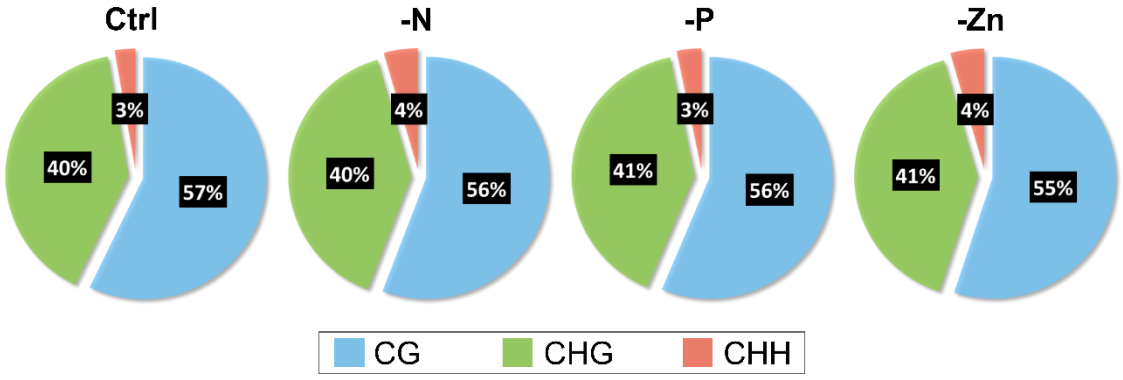

Figure 15: Contribution of each context methylation to the total methylation level.

This showed that in all treatments the same relative amount of methylation was lost in each context, so that the contribution of each context to the total methylation stayed the same.

As isolated, individual base methylation changes appear to have little functional relevance, only strongly differentially methylated regions (DMRs) were considered. DMRs were defined as regions of 50 to 500bp length, containing at least 4 cytosines and not less than 4 reads per cytosine, which differ by 40% or more in methylation, with a p-value of ≤ 0.01.

In agreement with the massive loss of methylation in -N and -Zn and minor methylation losses in -P, the smallest number of DMRs between control and deficiency samples was determined for -P (**Figure 16**). Surprisingly, in -Zn a lot more DMRs were found than in -N even though -N lost more methylation than -Zn. Most DMRs were present in the CG context, in total 2762 DMRs in -Zn, 1655 DMRs in -N and 461 DMRs in -P. In the CHG context, a smaller number of DMRs was identified for all samples and the lowest number still in -P.

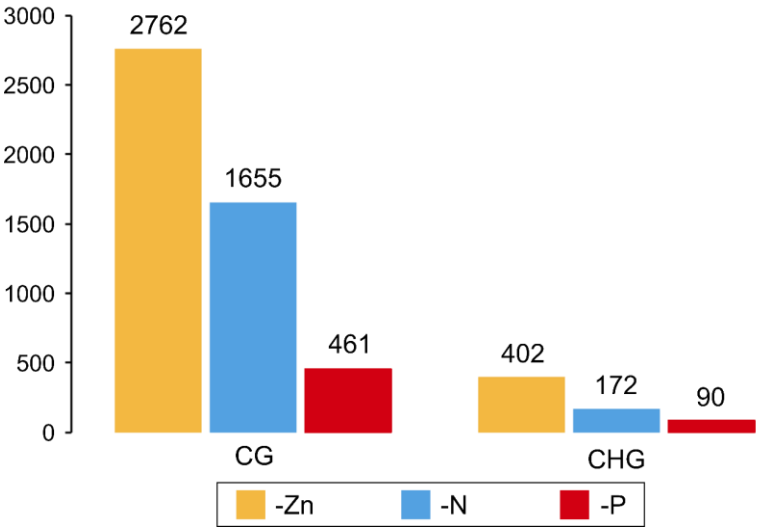

Figure 16: DMR count in CG and CHG context.

In contrast to CG and CHG, not a single DMR between control and treatment samples was encountered in the CHH context for -N and -P and for -Zn only 3 DMRs were found. To investigate if the conditions for defining a DMR were too strict to find DMRs in CHH context, less strict DMR criteria were tested (**Table 7**).

Table 7: Number of DMRs resulting from strict and loosened criteria defining a DMR.
DMRs with 40% (strict) and 10% (loose) methylation difference between control and treatment samples.

	-N		-P		-Zn	
	Strict	**Loose**	**Strict**	**Loose**	**Strict**	**Loose**
CG	1655	5933	461	1290	2762	10036
CHG	172	1301	90	310	402	2897
CHH	0	6	0	2	3	41

Strict criteria:	**Loosened criteria:**
≥ 40% Difference	≥ 10% Difference
≥ 4 Cytosines	≥ 3 Cytosines
≥ 4 Reads/Cytosine	≥ 3 Reads/Cytosine
50-500bp length	50-500bp length

Here, a minimum of 3 cytosines with at least 3 reads per cytosine and a minimal methylation difference of only 10% were used as criteria. But even under these conditions only 6 DMRs for -N and 2 for -P in CHH context were identified and for -Zn 41 DMRs after all. DMR numbers in CG and CHG contexts increased between 2.8 and 7.6-fold in the samples. Still the number of CHH DMRs was negligibly small, so it was decided to stick to the stricter DMR criteria to consider most severely affected chromosomal regions, which in previous studies were associated with substantial transcriptional differences (Secco et al., 2015).

As in all deficiency samples overall methylation was decreased in CG and CHG contexts, most DMRs were hypomethylated, especially in -N and -Zn. Here, about 92% to 98% of all DMRs were hypomethylated (**Table 8**). In -P a higher amount of

hypermethylated DMRs occurred, still leaving about 76% to 77% of DMRs hypomethylated.

Table 8: Percentage of DMRs being hypomethylated.

	-N	-P	-Zn
CG	97.70	77.22	97.10
CHG	92.35	75.56	93.03

When comparing in which genomic feature (transposable element, gene, promoter or spanning promoter and gene) DMRs (between control and deficiency samples) were located, for all samples and both CG and CHG contexts, by far the most DMRs were found to be positioned within TEs, namely more than 86% (up to 93%) for each sample (**Figure 17**). The lowest number of DMRs was located spanning both promoter and gene with only 1-2% in both contexts for -N and -Zn and none of the DMRs for -P. DMRs located in promoters were also relatively rare with 2-5% in the samples. DMRs in genes occurred in 4-9% in CG and CHG contexts in the different samples.

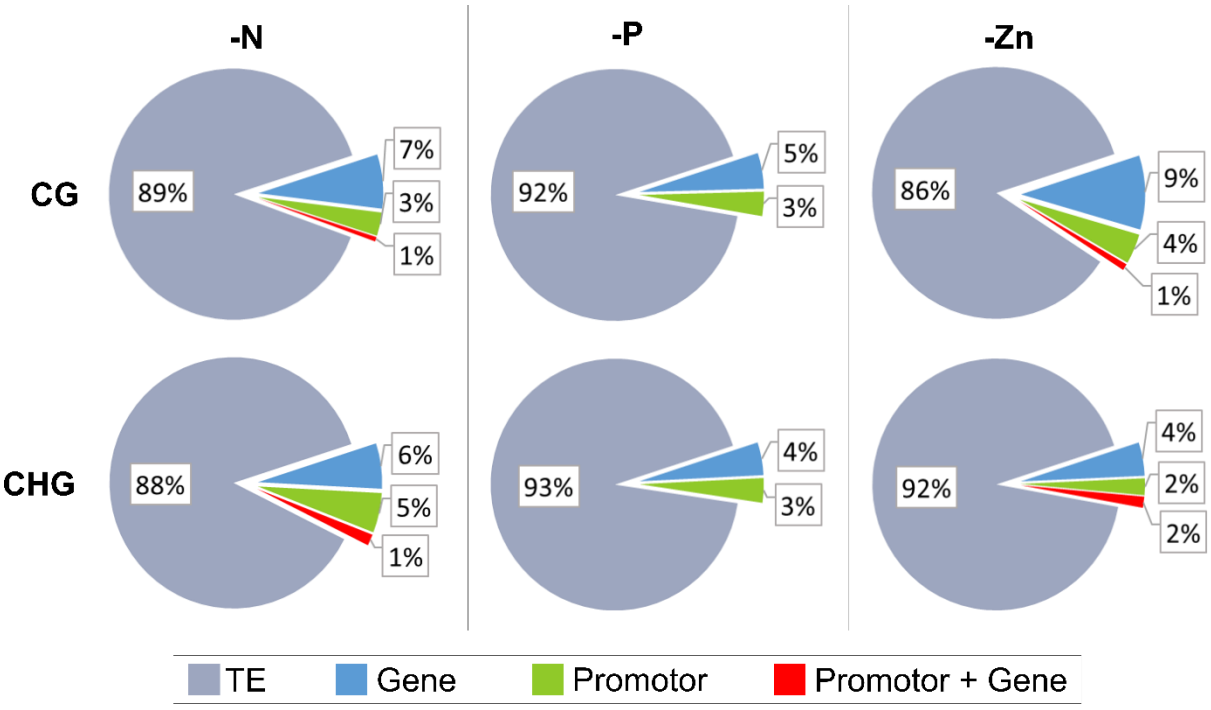

Figure 17: Percentage of DMRs located in transposable elements, promoters, genes or spanning promoter and gene.

To see if the methylation difference between each treatment and the control was bigger than natural variation between biological replicates, the number of DMRs between two random replicates of one sample was determined as well (**Table 9**). As expected, much smaller numbers of DMRs were found between replicates within one sample than when comparing control with treatment samples, ranging between 3.7fold to 45fold more DMRs found between treatments and control than within samples.

Table 9: Number of DMRs found between two replicates of one sample.

	Ctrl	-N	-P	-Zn
CG	14	39	13	393
CHG	2	9	2	110
CHH	0	0	0	1

4.3 Evaluation of RNA-sequencing and deficiency-regulated transcriptomes

Nutrient deficiencies rapidly alter gene expression in roots, but many initially strongly regulated genes abate to initial levels after some days, while a minority persists being different, often associated with developmental and metabolic changes under deficiency. It was aimed to capture the transcriptomic differences compared to the control after prolonged nutrient deficiency at the same point in time when the methylome analyses were made. The alignment rate for control, -N and -P samples to the reference genome was quite similar being around 90% for -P and 92% for control and -N (**Table 10**). Merely for -Zn it was lower with about 80%. Still, the alignment rate of all samples was sufficiently high to use the data in downstream analyses.

Table 10: Alignment rate of RNA-sequencing samples.
Values are averaged among replicates and shown in millions.

	Control	- N	- P	-Zn
Paired reads	28.0	27.9	27.7	27.7
Unaligned reads	2.3	2.7	2.3	5.7
Multiply aligned reads	2.7	2.5	2.6	2.5
Uniquely aligned reads	23.0	22.7	22.9	19.5
Alignement rate	**91.8%**	**90.3%**	**91.8%**	**79.6**

After alignment, transcripts were assembled and differentially expressed genes were determined. Through a principal component analysis, an insight into factors influencing the gene expression variance in the samples could be gained. The PCA was conducted with the FPKM values of -N, -P and -Zn. The PCA showed that the first two principal components comprised 95% of all samples' variance (**Figure 18**).

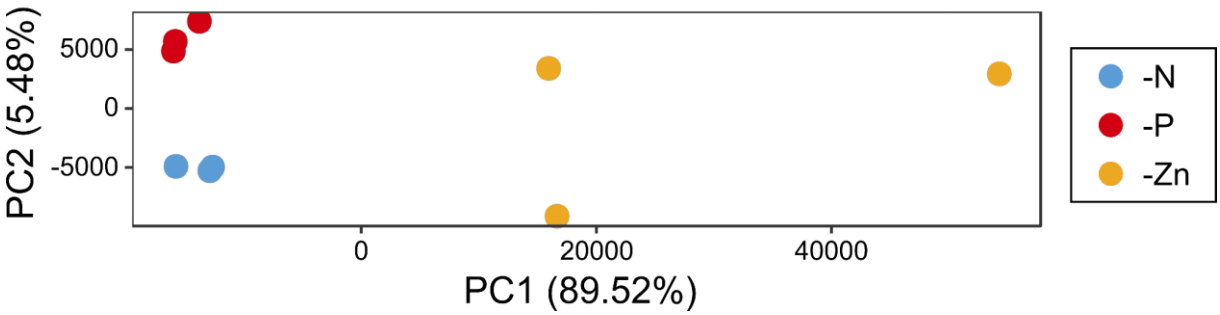

Figure 18: Principal component analysis of gene expression data.
PCA was conducted on FPKM values from RNA-sequencing.

Almost 90% were due to variance between -Zn replicates and between -Zn and the other two samples. About 5% were due to variance between -N and -P. The replicates of the samples did not overlap between samples and therefore the replicates of each sample could be grouped together. The high variance within -Zn in contrast to variance between -N or -P replicates might have been caused by the critical stress level in the -Zn plants.

A similar number of significantly differentially expressed genes (DEGs) was found for -P and -N samples, with -N having 7498 DEGs and -P 8208 DEGs of the altogether

evaluated 39469 *Zea mays* genes (**Figure 19A**). Even though in -Zn most DMRs were found, the sample had the smallest number of DEGs (4807). In each sample, similar proportions of up- and down-regulated DEGs were detected, namely roughly 50% of each. Considering the total amount of expression of all genes combined, the deficiency samples showed a slightly lower total expression than the control. The level in -N, -P and -Zn was reduced by about 2%, 6% and 3%, respectively, compared to the control. Looking for overlapping DEGs between the treatments showed that 1637 genes were differentially expressed in all three treatments (**Figure 19B middle**).

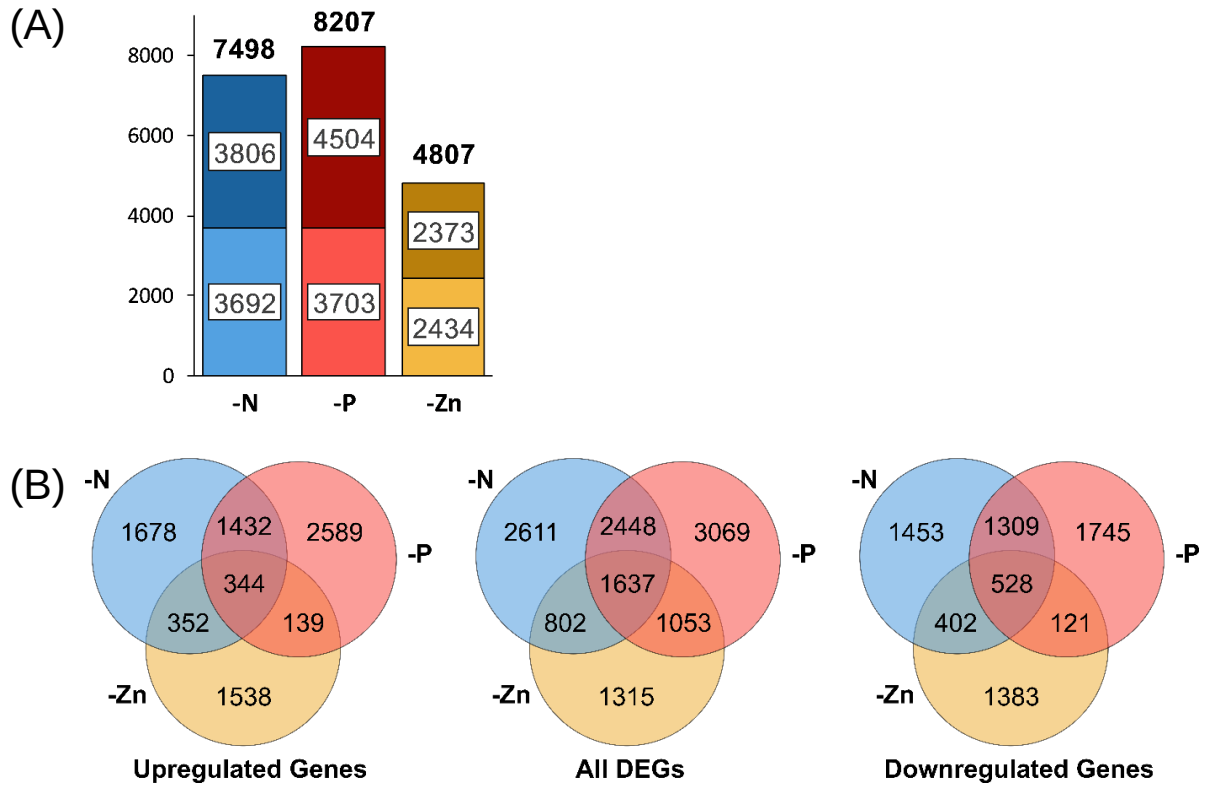

Figure 19: Number of DEGs for each treatment and amount of overlap between samples.
(A) Number of DEGs upregulated (upper part of bars) and downregulated (lower part of bars) between deficiency treatments and control. **(B)** Venn diagrams showing number of genes which were differentially expressed only in one of the treatments, in all or in two of them. Left: only upregulated genes, right: only downregulated genes, middle: all DEGs independent of up- or downregulation.

Of these overlapping genes, 872 genes were either down- or upregulated in all treatments while 765 genes were regulated in the contrasting direction in one treatment

than in the other two treatments. -N possessed 2611 genes which were differentially expressed only in this treatment, while 3069 genes were differentially expressed only in -P and 1315 genes only in -Zn. The shared fraction of DEGs between two of the treatments was always more or less equal for down- or upregulated genes, so there was no general bias for down- or upregulated genes being shared by any two treatments.

As CG methylation was massively lost in -N and -Zn, it was assumed that genes coding for maintenance methylation enzymes as well as enzymes involved in RNA-directed DNA methylation were down-regulated in -N and -Zn while demethylating enzymes might be up-regulated. This was partly substantiated by the RNA-seq data, although all methylation-related genes were expressed at relatively low levels. The majority of enzymes involved in maintenance methylation was either unchanged or downregulated in -N and -Zn while these genes were weakly upregulated or unchanged in -P (**Figure 20**).

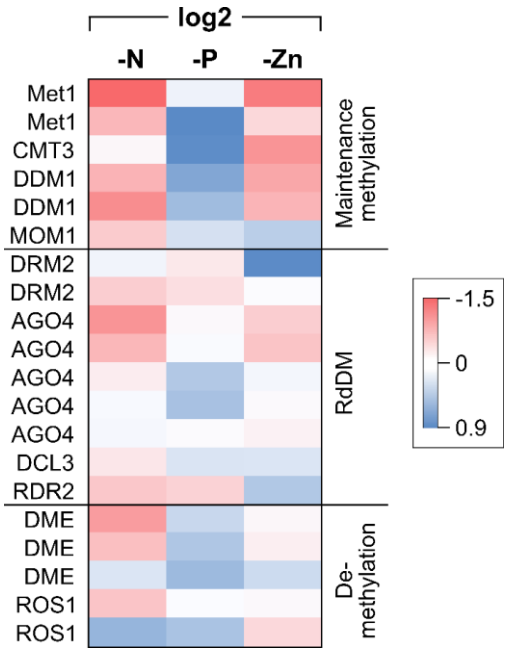

Figure 20: Color code for expression of methylating and demethylating enzymes.

For enzymes involved in RdDM -N showed mainly downregulation or no change while in -P and -Zn these genes were either unchanged or weakly up- or downregulated. Considering the demethylating enzymes, DME and ROS1 enzymes were both up- and downregulated in -N but they were mainly weakly upregulated in -P while the genes showed almost no change at all in -Zn.

Within the -N samples, crucial N deficiency-regulated marker genes were found to be differentially expressed. For example, genes encoding high affinity nitrate uptake systems (nitrate transporter 2 class), as well as high affinity glutamate-ammonia ligases (=glutamine synthetases) were substantially up-regulated (**Table 11**). By contrast, three genes encoding nitrate reductases were massively down in -N, in agreement with their common strong nitrate-regulated gene expression (Schluter et al., 2012). Likewise, key high affinity phosphorus uptake-related genes were upregulated in -P, namely inorganic phosphate transmembrane transporters 1;4 (PHT1;4) and phosphatases, of which some may be released from roots for mobilizing organic P (**Table 12**). Furthermore, genes encoding SPX domains, which are components of many proteins like phosphate transporters and signaling proteins (Wild et al., 2016) were upregulated. SPX domains seem to help sense limited P amount and aid in P starvation responses (Duan et al., 2008; Wild et al., 2016). Additionally, genes encoding proteins needed for phosphorus-independent bypass glycolysis reactions were upregulated, like phosphoenolpyruvate carboxylase (PEPC) and sucrose-phosphate synthase (SPS). Finally, many genes encoding proteins involved in lipid homeostasis and metabolism, tentatively in readjusting membrane lipids to potentially reduced phospholipid levels, were also up-regulated, among them being Lipase class 3 family proteins, UDP-sulfoquinovose:DAG sulfoquinovosyltransferase and UDP-galactosyltransferase.

Table 11: Nitrogen deficiency-regulated differential gene expression.

Gene ID	Annotation	FPKM Ctrl	FPKM -N	Log_2 FC
GRMZM2G010280	Nitrate Transporter 2:1	170.3	674.5	1.99
GRMZM2G010251	Nitrate Transporter 2:1	115.2	244.5	1.09
GRMZM2G455124	Nitrate Transporter 2:5	3.6	349.2	6.60
GRMZM5G878558	Nitrate Reductase 1	251.0	1.5	-7.35
GRMZM2G568636	Nitrate Reductase 1	167.8	45.2	-1.89
GRMZM2G102959	Nitrate Reductase 1	243.3	1.9	-7.03
GRMZM2G036464	Glutamate-ammonia ligase	121.9	680.1	2.48
GRMZM5G872068	Glutamate-ammonia ligase	240.5	660.8	1.46

Typical zinc-regulated genes were found in -Zn where several genes coding for zinc uptake systems (zinc ion transmembrane transporters, ZIP) were upregulated (**Table 13**). Additionally, nicotianamine synthase (NAS), synthesizer of nicotianamine, which aids in uptake and transportation of zinc and other heavy metals, was mostly upregulated during zinc deficiency, especially NAS4. As zinc is needed for detoxification of superoxide radicals via superoxide dismutase, this enzyme was downregulated under zinc deficiency. Carbonic anhydrase, which catalyzes CO_2 hydration, was also reduced in -Zn. There was an increase in purple acid phosphatases which helps maintaining inorganic phosphate metabolism (Bharti et al., 2014). Expansin, however, which plays a role in plant cell growth, was downregulated under -Zn. On the other hand, nodulin proteins, which are involved in symbiotic nitrogen fixation (Verma et al., 1986), were both up- and downregulated under -Zn.

Table 12: Phosphorus deficiency-regulated differential gene expression.

Gene ID	Annotation	FPKM Ctrl	FPKM -N	Log_2 FC
GRMZM2G326707	Inorganic phosphate transmembrane transporter, PHT1;4	73.0	305.5	2.07
GRMZM2G154090	Inorganic phosphate transmembrane transporter, PHT1;4	6.7	695.6	6.69
GRMZM2G112377	Inorganic phosphate transmembrane transporter, PHT1;4	1.4	271.3	7.64
GRMZM2G069542	phosphoenolpyruvate carboxylase	92.7	154.5	0.74
GRMZM2G074122	phosphoenolpyruvate carboxylase	42.7	73.6	0.79
GRMZM2G110714	phosphoenolpyruvate carboxylase	3.2	22.9	2.83
GRMZM2G008507	Sucrose-phosphate synthase	1.2	77.9	6.02
GRMZM2G047995	Lipase class 3 family protein	4.0	87.0	4.45
GRMZM2G169562	Lipase class 3 family protein	2.9	27.3	3.23
GRMZM5G829946	Senescence-related gene 3, glycerophosphodiester phosphodiesterase	0.3	159.3	8.92
GRMZM2G064962	Glycerophopsphoryl diester phosphodiester family protein	19.2	108.0	2.49
GRMZM2G315848	Protein nucleotide pyrophosphatase/phosphodiesterase	6.7	65.8	3.29
GRMZM2G477503	Sulfoquinovosyldiacylglycerol 2	6.5	348.0	5.75
GRMZM2G141320	1,2-diacylglycerol 3-beta-galactosyltransferase/ UDP-galactosyltransferase	0	159.8	-
GRMZM2G152447	Acid phosphatase/ protein serine/threonine phosphatase	1.0	318.8	8.34
GRMZM2G138756	Acid phosphatase/ protein serine/threonine phosphatase	0.1	17.7	8.00
GRMZM5G836174	Phosphatase	0.5	1991.8	11.83
GRMZM2G015908	Phosphatase	3.9	309.9	6.30
GRMZM2G021106	Phosphatase	0.4	48.1	6.81
GRMZM2G171423	SPX domain gene 2	1.0	84.6	6.46
GRMZM5G805389	SPX domain gene 3	3.3	1246.0	8.57
GRMZM2G065989	SPX domain gene 3	2.5	1008.0	8.65
GRMZM5G828488	SPX domain gene 3	0.5	399.5	9.65

Table 13: Zinc deficiency-regulated differential gene expression.

Gene ID	Annotation	FPKM Ctrl	FPKM -Zn	Log_2 FC
GRMZM2G111300	Zinc ion transmembrane transporter, ZIP1	68.3	255.7	1.90
GRMZM2G045849	Zinc ion transmembrane transporter, ZIP1	52.9	106.0	1.02
GRMZM2G064382	Cation transmembrane transporter/ copper ion transmembrane transporter, ZIP4	8.3	154.6	4.21
GRMZM2G015955	Cation transmembrane transporter/ copper ion transmembrane transporter, ZIP4	114.9	195.9	0.77
GRMZM2G047762	Cation transmembrane transporter/ metal ion transmembrane transporter, ZIP5	26.2	134.9	2.37
GRMZM2G093276	Iron ion transmembrane transporter/ zinc ion transmembrane transporter, IRT2	20.6	119.6	2.54
GRMZM2G106928	Superoxide dismutase	58.0	25.6	-1.18
GRMZM2G175728	Superoxide dismutase	11.7	3.2	-1.89
GRMZM2G478568	Nicotianamine synthase 4	19.2	45.1	1.23
GRMZM2G050108	Nicotianamine synthase 4	35.5	186.7	2.40
GRMZM2G312481	Nicotianamine synthase 4	0.4	2.2	2.64
GRMZM2G385200	Nicotianamine synthase 4	1.5	8.5	2.49
GRMZM2G034956	Nicotianamine synthase 4	5.0	0.4	-3.60
GRMZM2G348512	Carbonic Anhydrase 2	230.8	125.3	-0.88
GRMZM2G046924	Carbonic Anhydrase 1	89.2	54.1	-0.72
GRMZM2G073860	Purple acid phosphatase 10	7.8	17.9	1.19
GRMZM2G134054	Purple acid phosphatase 15	3.90	10.9	1.48
GRMZM2G133322	Nodulin MtN3 family protein	1.2	78.3	6.07
GRMZM2G060974	Nodulin MtN3 family protein	17.5	0.3	-5.77
GRMZM2G173669	Nodulin MtN3 family protein	75.2	1229.6	4.03
GRMZM2G179349	Nodulin MtN3 family protein	7	79.2	3.51
GRMZM2G139834	Nodulin MtN21 family protein	29.1	2.6	-3.51
GRMZM2G467893	Nodulin family protein	11.1	128.7	3.54
GRMZM2G001035	Nodulin	70.3	1.7	-5.34
GRMZM2G173826	Expansin-A12 precursor	15.4	1.7	-3.16
GRMZM2G450546	Expansin-A19 precursor	6.3	0.3	-4.51
GRMZM2G021427	Expansin-B3 precursor	20.6	2.3	-3.15
GRMZM2G327266	Expansin-B11 precursor	39.8	4	-3.31

4.4 Correlation between DNA methylation and transcriptional changes

RRBS and RNA-sequencing results were used to identify possible correlations between nutrient-deficiencies, methylation adaptation and accompanying gene transcription changes.

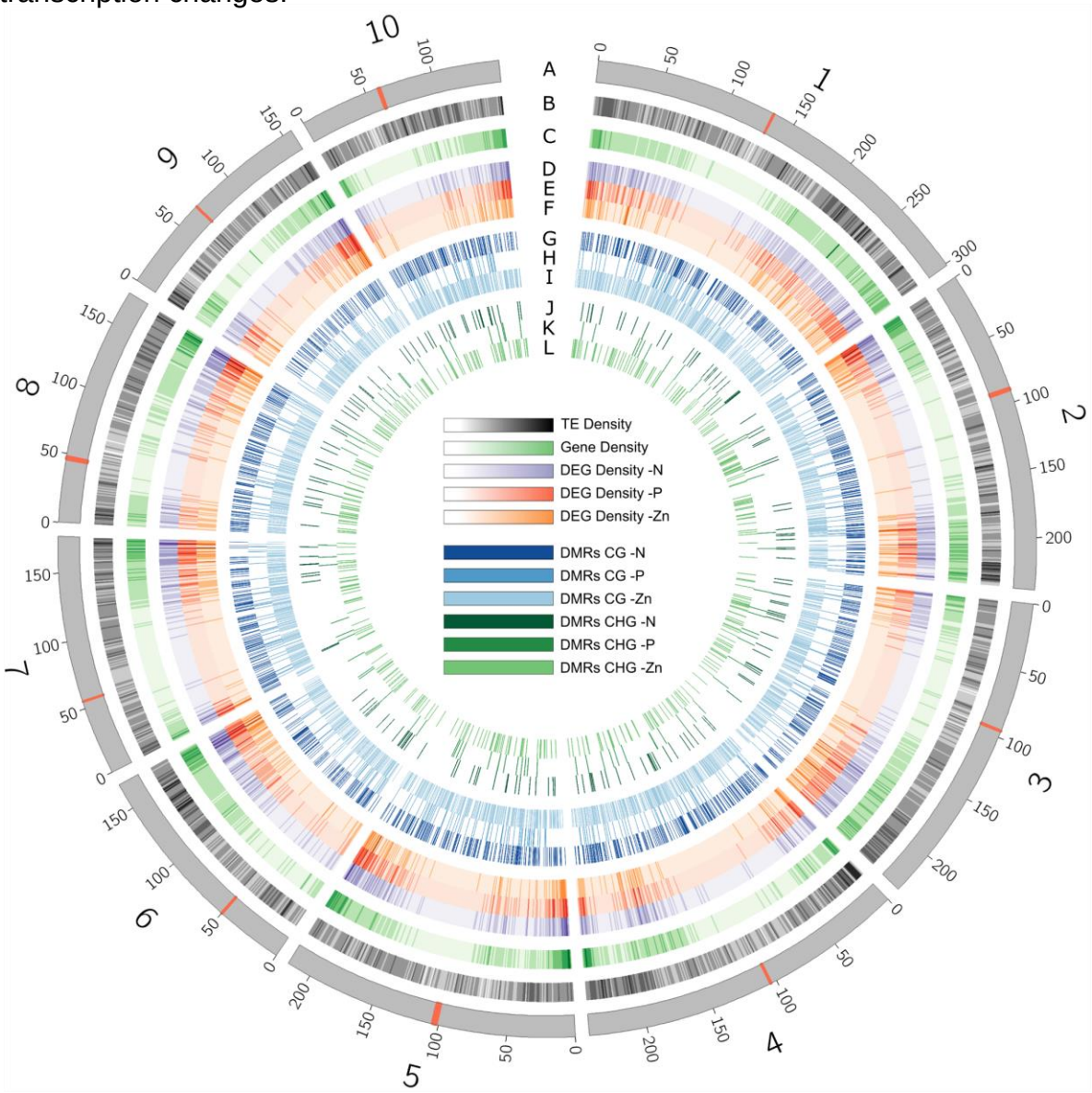

Figure 21: Distribution of different features across the chromosomes of the maize B73 genome.
(A) Chromosomes with the centromeres shown as red bands. **(B)** Density of transposable elements. **(C)** Density of genes. **(D)** Density of DEGs in -N. **(E)** Density of DEGs in -P. **(F)** Density of DEGs in -Zn. **(G)** Distribution of CG DMRs in -N. **(H)** Distribution of CG DMRs in -P. **(I)** Distribution of CG DMRs in -Zn. **(J)** Distribution of CHG DMRs in -N. **(K)** Distribution of CHG DMRs in -P. **(L)** Distribution of CHG DMRs in -Zn.

The entire chromosomal distribution of TEs, Genes, DEGs and DMRs in the whole maize genome is shown in **Figure 21** including the 10 chromosomes in which the centromeric regions (Wolfgruber et al., 2009) are shown as red bands.

Transposable elements were relatively equally distributed over each chromosome, while the gene density was clearly enriched towards the terminal ends of each chromosome arm and depleted in centromeres and centromere-flanking regions. In agreement with higher gene density at the outer chromosomal ends, DEGs in -N, -P and -Zn were enriched in these regions. In contrast to that, CG and CHG DMRs were relatively uniformly distributed across each chromosome. The large overlap of TEs with DMRs is consistent with the fact that most DMRs were positioned in TEs.

As differential methylation in a gene or its promoter region might influence the expression of that gene, the percentage of differentially expressed genes containing a DMR in their gene body or promoter was determined (**Figure 22**).

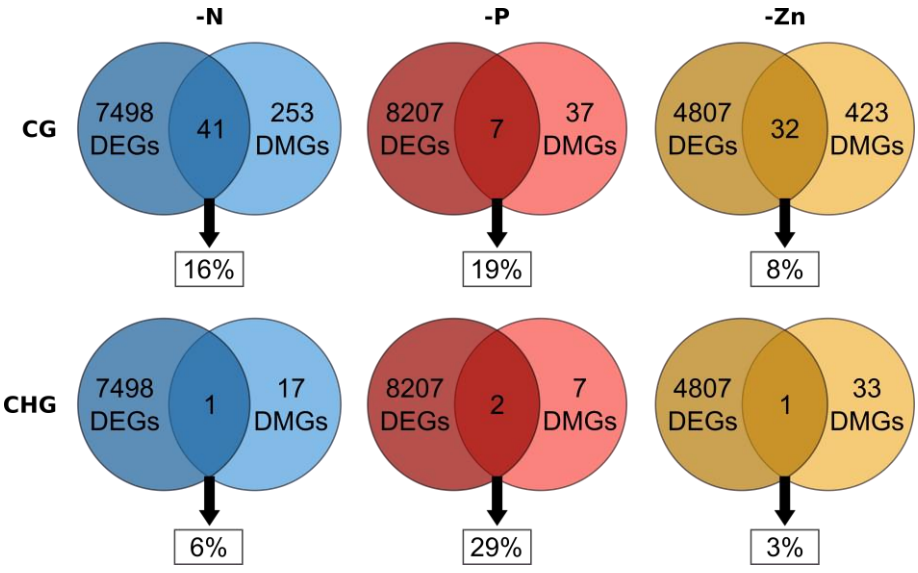

Figure 22: Number of genes being differentially expressed and/or methylated. Percentages are relative to differentially methylated genes (DMGs).

In the CG context, 41 of the 253 differentially methylated genes were also differentially expressed in -N, being 16% of the genes with DMRs. In -P, 19% of the differentially

methylated genes were differentially expressed. For -Zn 32 of the 423 genes with a DMR were also differentially expressed, comprising 8%. In the CHG context, only one (-N and -Zn) or 2 genes (-P) were differentially methylated and expressed at the same time, comprising 6%, 29% and 3% for -N, -P and -Zn, respectively.

For the examination of the statistical significance of these observations, only genes were taken into account that were covered by the reduced representation methylome by at least 500 base pairs. For the analysis of differentially methylated TEs and their potential influence on the expression of the closest gene, however, no such cutoff was applied (TEs only had to be covered by at least one bp), to avoid the loss of many of the often very short TEs from the analysis. A significant correlation (at 5 % level) of methylation and gene expression was suggested by Fisher's exact test for CG methylation in TEs next to DEGs for -N and -P with a p-value of 0.003 and 0.009, respectively (**Table 14**). For -Zn, on the other hand, there was no significance for a correlation between differentially methylated TEs and the expression of the closest gene. Instead, significant correlations were suggested by p-values of 0.005 and 0.023 for differentially methylated genes and promoters, respectively, and the expression of the according gene.

Table 14: Results of Fisher's exact test for determination of correlations between differential methylation in different genome features and gene expression.

	-N		-P		-Zn	
	CG	**CHG**	**CG**	**CHG**	**CG**	**CHG**
DMR in gene	0.059	0.220	0.440	0.552	**0.005**	0.347
DMR in promoter	0.579	1.000	0.231	0.414	**0.023**	0.381
DMR in TE	**0.003**	0.594	**0.009**	0.666	0.699	0.483

To examine if there were linear correlations between differential methylation and gene expression, scatter plots were applied for differentially methylated TEs whose closest gene was differentially expressed as well as for differentially expressed genes which had a DMR in their promoter or gene body (**Figure 23**).

The predominant reduced methylation was frequently involved in up- or down-regulated gene expression, without preference, in all treatments and for both differentially methylated promoters/gene bodies (**Figure 23A**) and differentially methylated TEs (**Figure 23B**).

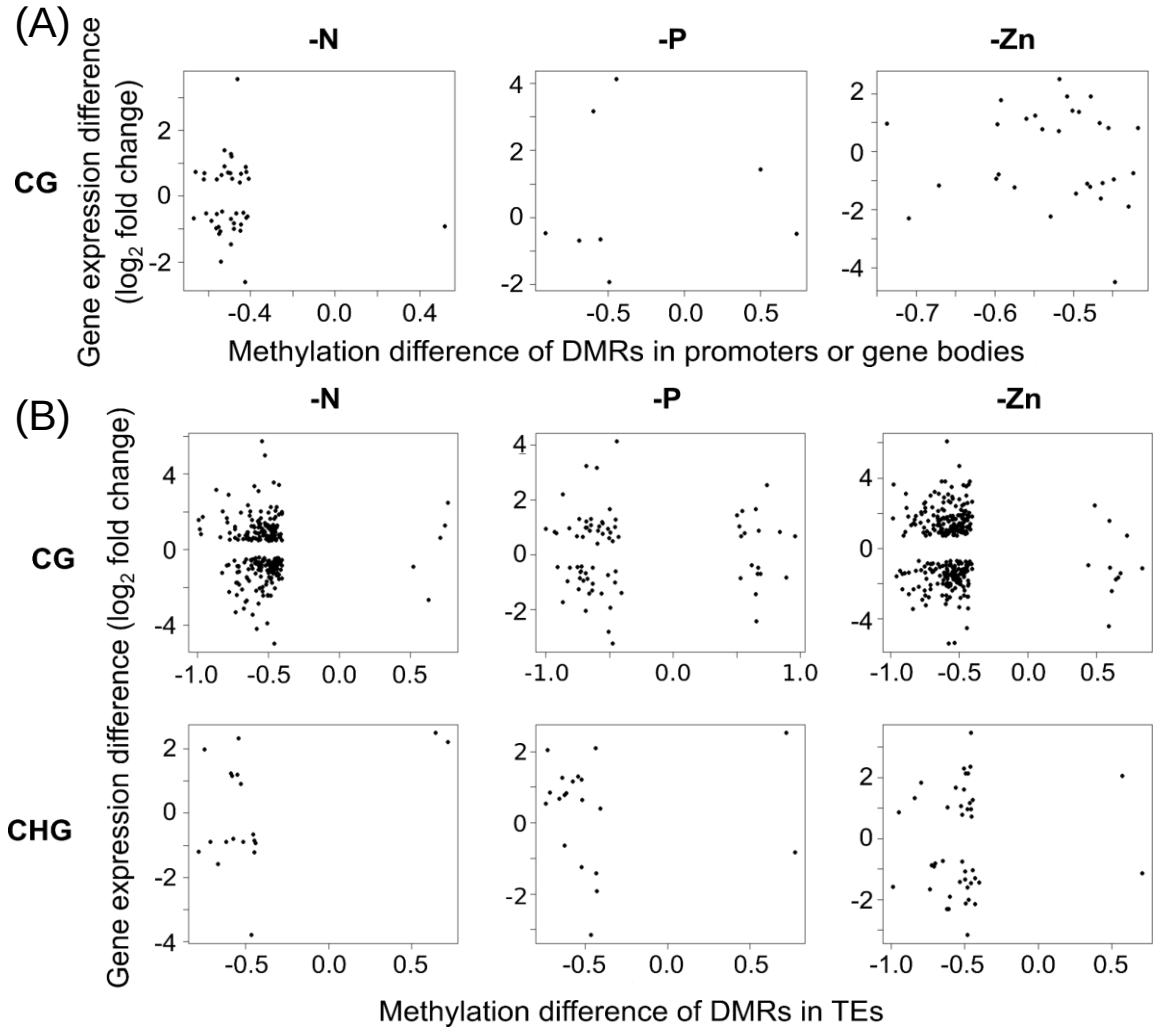

Figure 23: Scatterplots for investigation of linear correlations between methylation and expression of significantly differentially expressed genes.
(A) The methylation difference of DMRs in gene promoters or gene bodies on the x-axis against the expression difference of the genes on the y-axis. **(B)** The methylation difference of DMRs in TEs on the x-axis against the expression of the closest gene on the y-axis.

Even though there were significant correlations in the CG context found for differentially expressed TEs and the gene expression of the closest gene in -N and -P and a significant correlation between differentially methylated genes and their gene

expression in -Zn, no linear correlation could be observed in any context. The same holds true for the few hypermethylated TEs, mostly found in the -P CG context.

A similar result can be seen if not only significantly differentially expressed genes, but all genes are considered (**Figure 24**).

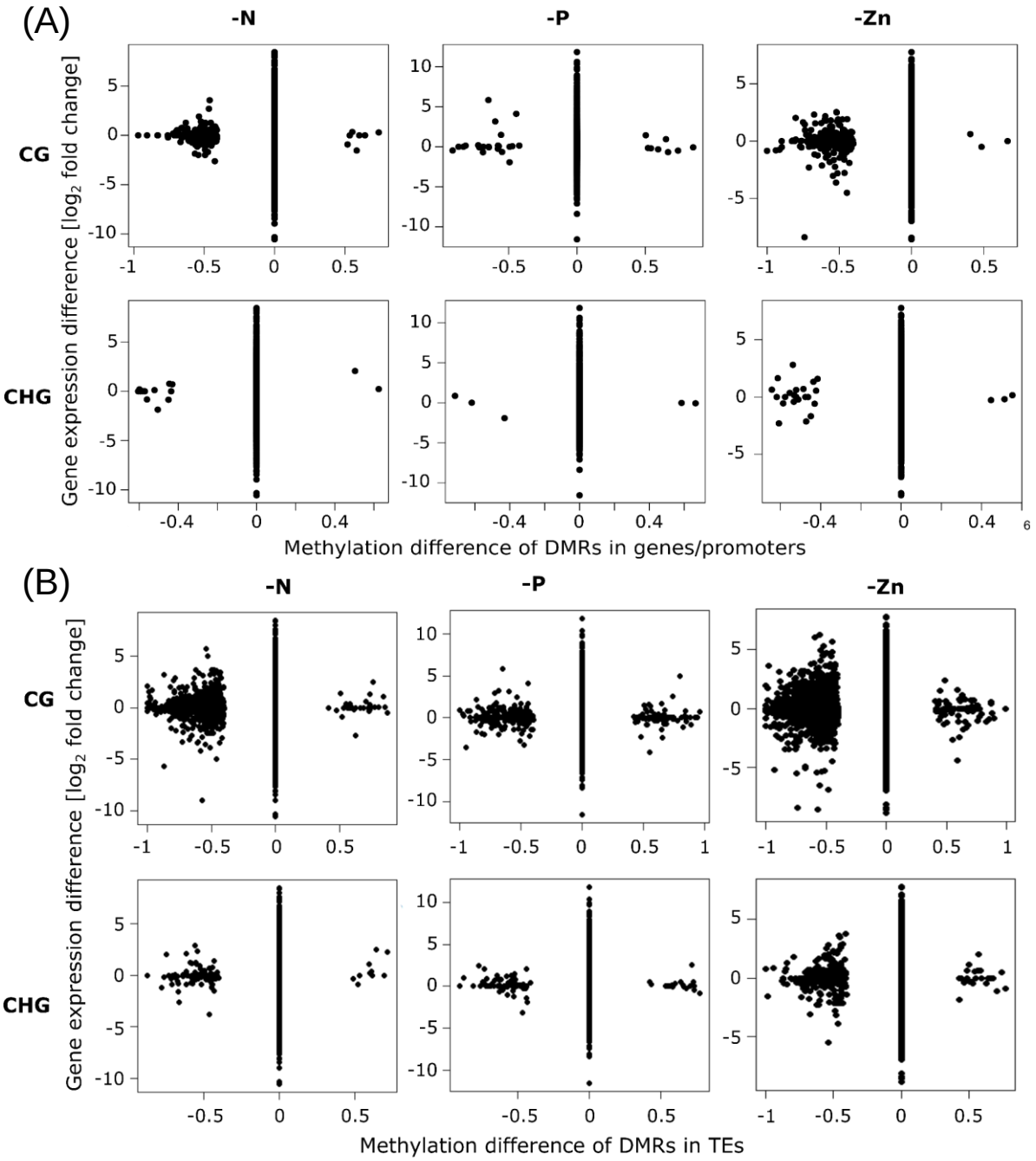

Figure 24: Scatterplots for investigation of linear correlations between methylation and expression difference of all genes.
(A) Genes of which some are differentially expressed and/or contain a DMR in the gene body/promoter. **(B)** Genes of which some are differentially expressed and/or contain a nearby differentially methylated TE.

Among the genes that were differentially expressed and possessed a DMR in their gene body, promoter or nearby TE, were some that belong to potentially nutrient-specific deficiency-regulated genes. The position of DEGs, together with their gene structure and corresponding DMR is shown for some examples in **Figure 25**.

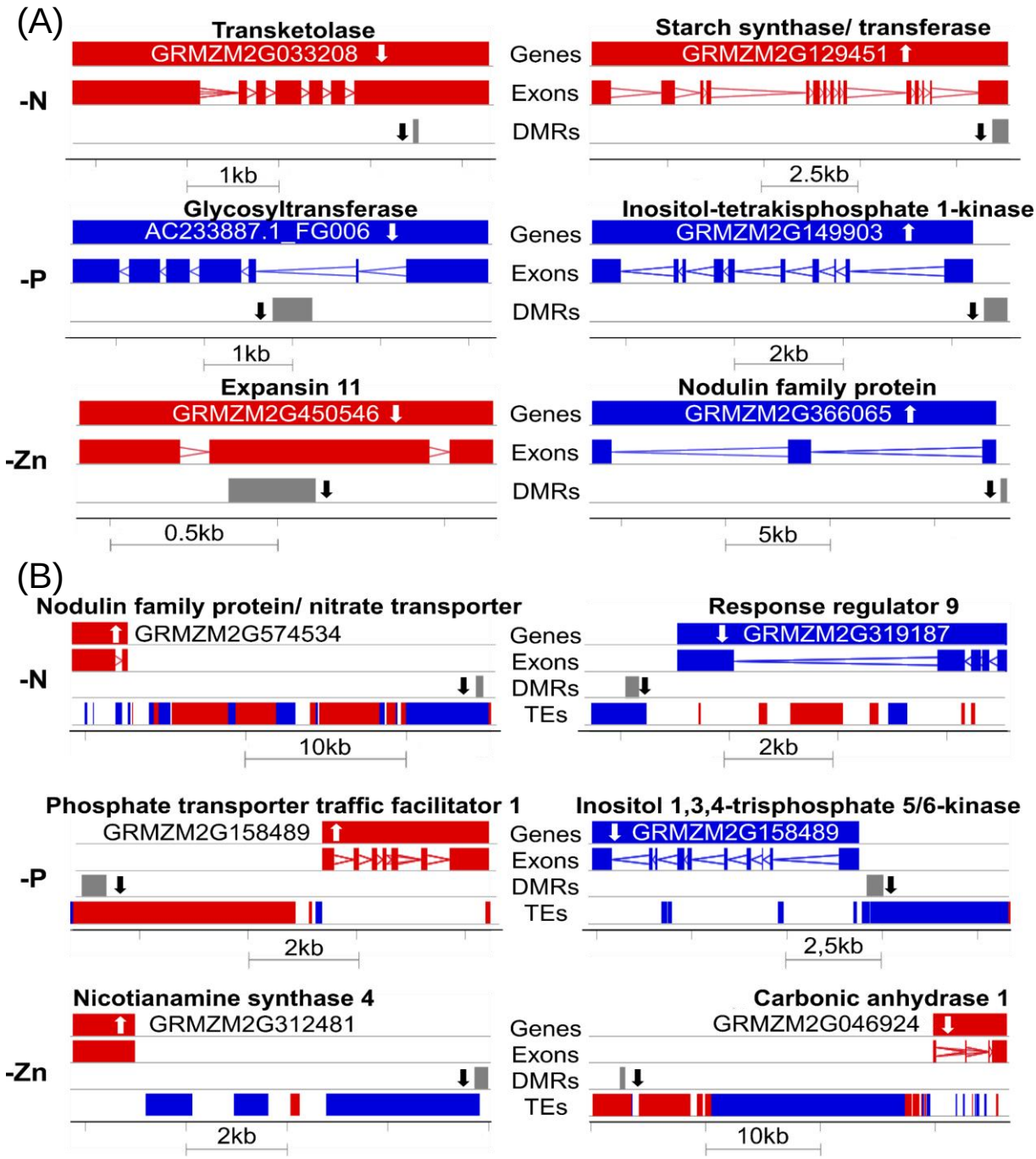

Figure 25: Examples for correlation between DNA methylation and gene expression.
(A) DMRs in gene bodies/promoters of differentially expressed genes. **(B)** DMRs in TEs nearby differentially expressed genes.

Examples for genes that were both differentially methylated and expressed are shown in (**Figure 25A**). A down-regulated transketolase and an up-regulated starch synthase were both hypomethylated in -N. A hypomethylated glycosyltransferase was decreased in expression, while a hypomethylated Inositol-tretrakisphosphate 1-kinase was higher expressed in -P. In -Zn samples an expansin 11 was downregulated while a nodulin family protein was upregulated even though both were hypomethylated. In the examples for differentially methylated TEs near to differentially expressed genes (**Figure 25B**) a putative induced nitrate transporter gene close to a hypomethylated transposable upstream element in -N is shown. By contrast, a hypomethylated TE was close to a down-regulated gene of response regulator 9. Similar cases are also shown for -P. Hypomethylation in TEs was associated with up-regulation of phosphate transporter traffic facilitator 1 but downregulation of inositol 1,3,4-trisphosphate 5/6-kinase. In -Zn a nicotianamine synthase 4 was upregulated and a carbonic anhydrase downregulated while each of them were located near a hypomethylated TE. Generally, although most methylation changes were hypomethylations, both up- and downregulation of genes occurred. Taken together, a minor correlation between differentially methylated TEs and the expression of closely neighbored genes was observed in -N and -P, while gene expression and direct methylation were remarkably independent of each other in the two samples. In -Zn, it was the other way around.

As expression of TEs may be induced by hypomethylation, expression of the TEs in the dataset was also checked for. Within all detected transcripts, only 487 sequences were annotated to contain transposable element sequences. However, the number of expressed TEs did not change in the deficiencies, while the average expression level

of these transcripts was moderately increased by 25% in -N, and by 22% in -P, compared to the control. In -Zn, overall TE expression was reduced by 9%.

4.5 Amount of small RNAs

As small RNAs play a role in the RNA-directed DNA methylation pathway, which is especially important for CHH but also for CHG and CG contexts, it was suspected that the number of small RNAs might be decreased in the samples that lost a lot of methylations. This assumption did not prove to be totally true. Even though -N and -Zn samples, in which much methylation was lost, did contain the smallest number of small RNAs in relation to total RNA, -Zn contained much less than -N, despite that the methylation loss was bigger in -N than in -Zn (**Figure 26**). Additionally, -P also lost a small amount of DNA methylation but has a higher ratio of small RNAs to total RNA than the control, with -P containing 12.9% and the control containing 9.7%.

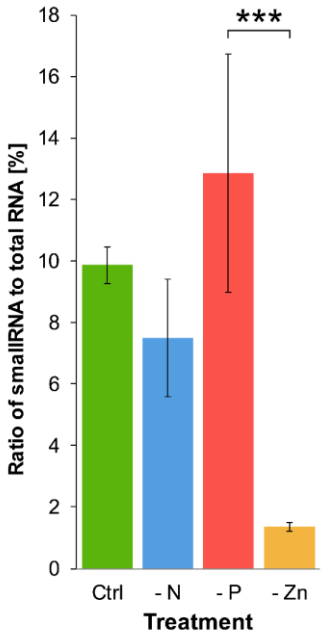

Figure 26: Amount of small RNAs.
Error bars = standard deviation.

5 Discussion

DNA Methylation in plants has various functions which are by far not totally understood yet. Our intensive approach for investigating the influence of nutrient deficiencies on the methylation level in maize roots showed a very strong loss of methylation due to nitrogen and zinc deficiency, especially in the CG and CHG contexts. Interestingly, the loss of methylation in the two contexts was much less pronounced when growing the plants under lacking phosphorus.

The transcriptomes in the roots of the same plants showed some typical nutrient-specific adaptations in gene expression. Most changes in gene expression compared to the control were found in -N and -P samples and only a bit more than half as many in -Zn samples. With methylation loss being very strong in -N and -Zn, most expression change was expected in these two samples. With this not being the case, there was also only moderate correlation encountered between methylation changes and gene expression adaptation due to the nutrient deficiencies. This led to the assumption that DNA methylation changes not only increased plasticity by gene expression changes but DNA methylation loss also played a role in increasing genetic diversity in following generations by increasing the frequency of recombination events and transposon movements.

5.1 Maize growth performance and plant material

Previous research indicated that especially phosphorus deficiency strongly affects DNA methylation, but results from rice and *Arabidopsis* were little congruent. Here, the methylome and transcriptome of *Zea mays* roots grown under nitrogen-, phosphorus- or zinc-deficient conditions were compared. Growing the plants in a controlled

environment allowed us to investigate parallel methylation and transcriptional changes caused by a single nutrient deficiency, ruling out other environmental impacts. Phenotypic analysis of the plants as well as the nutrient analysis and the induction of typical starvation-induced genes confirmed that the plants were specifically stressed from lack of the intended nutrient. Some previous research suggested that most plant tissues do not vary tremendously in their DNA methylation, for example methylation in *Arabidopsis thaliana* between leaf and inflorescence (Schmitz et al., 2013), in rice between roots, shoots, endosperm and embryo (Zemach et al., 2010), and six of seven tissues in *sorghum* did not vary strongly (except endosperm tissue) (Zhang et al., 2011). In *Populus trichocarpa* one-third of the genome was found to be differentially methylated among seven different tissues (Vining et al., 2012) and up to 12% difference in methylated cytosines between root and shoot tissues was found in *Arabidopsis thaliana*, *Arabidopsis lyrata* and *Capsella rubella* (Seymour et al., 2014). This work focused on root tissues for analysis of methylome and transcriptome, as this is the most important plant organ for nutrient sensing and uptake.

5.2 Nutrient-specific methylome

One of our main interests was to find out if methylation adapts not only to environmental stresses, like lack of nutrients, in a general stress-related way, but also specifically depending on which nutrient is lacking. Maize root samples from plants grown on nitrogen or zinc deficiency experienced an immense loss of methylated cytosines, especially in the symmetrical contexts. Phosphorus deficiency samples on the other hand showed only a minor loss in the symmetrical contexts and no loss at all in the asymmetrical context. These differences of the methylation level in the nutrient deficiency samples indeed suggested a nutrient-specific adaptation in DNA

methylation rather than simply a general stress reaction. In addition, based on the phenotypes of the plants, -Zn treatment caused the highest stress level as the plants were the smallest and close to dying, while the phenotypes of -N- and -P-treated plants implicated only a minor difference in the stress level. Therefore, methylation loss exclusively due to stress level would give reason to expect strongest loss in -Zn samples and weaker losses in both -N and -P. The loss of methylation, however, was found to be even a bit stronger in -N than in -Zn.

The reduction in overall methylation, especially in the CG context, went along with DMRs being mostly hypomethylated as well. This was more pronounced in -N and -Zn samples, with more than 90% of CG and CHG DMRs lower methylated than the control. The weaker loss in methylation in -P resulted in 77% of CG and 76% of CHG DMRs being hypomethylated. This contrasts the situation in rice, belonging like maize to the family of Poaceae, where it was found that 84% of DMRs under phosphorus-starvation conditions were hypermethylated (Secco et al., 2015). Under -N almost no change in the overall methylation level occurred in rice (Kou et al., 2011) but hyper- and hypomethylated regions were found. In *Arabidopsis thaliana*, the overall methylation level of phosphorus-deficient plants was found to almost double within 17 days with the increase of methylation occurring in all contexts and 86% of the DMRs were hypermethylated (Yong-Villalobos et al., 2015). Only a limited amount of methylation changes in *Arabidopsis* under phosphorus deficiency was found by Secco et al., 2015.

As CHH methylation is placed *de novo* on the DNA and thereby believed to be more highly and faster adaptable to environmental conditions than CHG and CG contexts, it was assumed that DNA methylation would vary the most in this context between control and deficiency plants. Interestingly, the most changing context was CG, while

the CHH context was only slightly methylated in general and did not change enormously so that only a negligible number of DMRs was found in CHH context, even with relaxed criteria for defining a DMR. These findings were in accordance with Li et al., 2015 who also recognized low methylation in CHH context and only a very small number of DMRs compared to the symmetric contexts in 5 different maize inbred lines, including B73. The same discovery has been made by Eichten et al., 2013, who also found the CHH context of the inbred lines B73 and Mo17 low (<10%) methylated. In *Arabidopsis thaliana* a similar methylation level (1.7%) in CHH context was found (Law and Jacobsen, 2011) as in the maize plants in the experiments of this work (1.3% in control plants). On the other hand, a high amount of hypermethylation was found in the CHH context in rice due to phosphorus deficiency (Secco et al., 2015). These differences in DNA methylation changes under nutrient deficiencies might be caused by the different points in time of sample-taking or by different criteria defining DMRs, but they also suggest highly species-specific mechanisms (Roessler et al., 2016).

The changes in DNA methylation were present after 4 weeks of deficiency treatment, indicating long-term changes, though it is not clear whether the adaptation occurred soon after the start of the deficiency treatment and was upheld still after 4 weeks or if methylation kept changing until the samples were taken or if the changes started only after some time. In rice, a bigger change in DNA methylation levels occurred after 24 days of -P treatment than after 3 and 7 days (Secco et al., 2015), indicating a continuous adaptation of DNA methylation under prolonged deficiency conditions.

5.3 Nutrient-specific transcriptome

Today, there are several genes which are known to be regulated by a specific nutrient deficiency. Among them are high affinity transporters for a certain nutrient which are

upregulated to provide efficient uptake of small amounts present of this nutrient. For all of the tested nutrients, the upregulation of the particular transporters was found.

In contrast to nitrate transporters, nitrate reductases, which reduce nitrate to nitrite, were strongly downregulated in the -N samples which is confirmed by literature where the reduction was found for -N in maize and *Arabidopsis* as well (Menz et al., 2016; Schluter et al., 2012). For -P phosphatases were also found to be highly upregulated which is in accordance with research done in other plants and tissues (Aono et al., 2001; Li et al., 2012; Secco et al., 2015; Vance et al., 2003; Wang et al., 2014; Wu et al., 2003; Yong-Villalobos et al., 2016; Zhang et al., 2014; Zörb and Müller, 2015). SPX domain genes were also drastically upregulated. SPX was found to be upregulated under -P by other researchers already in a wide range of plants including maize, *Arabidopsis* and rice (Duan et al., 2008; Li et al., 2012; Secco et al., 2013, 2015; Wild et al., 2016; Yong-Villalobos et al., 2015) which is explained by proteins involved in P uptake and transport containing SPX domains (Wild et al., 2016). Other genes are less consistently up- or downregulated due to P-starvation-regulation. PEPC is often found to be differentially expressed under phosphorus stress. In this work, a slight upregulation of PEP carboxylases in the maize roots was found which was highly contrasted to the findings made in maize leaves under -P (Zhang et al., 2014). PEPC is involved in photosynthesis by regulating the concentration of CO_2. Photosynthesis takes place in the leaves which is probably the reason for the tissue-specific (root vs. shoot) difference in expression of PEPC following P deficiency. While nitrogen- and phosphorus-starvation reactions on the gene expression level are more or less well known today, the zinc deficiency response is less well investigated. Besides the zinc transmembrane transporters also nicotianamine synthase (NAS), which synthesizes the zinc transporting nicotianamine, was previously found to be upregulated

(Assunção, Schat, et al., 2010) which was confirmed in our samples for 4 out of 5 genes encoding NAS4. Carbonic anhydrase was slightly downregulated in the -Zn maize roots and was also found to be downregulated in spinach and rice plants (Broadley et al., 2011; Randall and Bouma, 1973). The upregulation of purple acid phosphatases under -Zn in maize was also found in wheat (Bharti et al., 2014). Other genes did show a less consistent expression. Van De Mortel et al., 2006 found nodulin-like proteins to be downregulated in *A. thaliana* roots after growth on -Zn. However, 4 nodulin family proteins were found to be highly upregulated while 3 nodulin proteins were downregulated in the -Zn maize roots in the here described work. However, expansins were found to be downregulated in Zn-deficient *Arabidopsis* roots (Van De Mortel et al., 2006) and they were also decreased in the -Zn maize root samples.

All in all, some genes seem to be differentially expressed in plants as a general stress response whereas other genes are changed specifically by deficiency of a certain nutrient. Additionally, there are genes which change their expression due to a specific nutrient deficiency in a highly plant- and tissue-specific way.

5.4 Limited correlation between DNA methylation and gene expression

Knowledge about DNA methylation adaptation to different nutrient-stresses and its influence on gene expression in maize is scant, as most research concerning these topics has been conducted in the model plant *Arabidopsis thaliana*. Since quite some time it is assumed that the possibly harmful activity of transposable elements is silenced by hypermethylation of transposons (Dowen et al., 2012; Slotkin and Martienssen, 2007; Tan, 2010; Tsaftaris et al., 2003; Yong-Villalobos et al., 2015). Increasing evidence now suggests that changing methylation in transposons might

also have an effect on gene expression regulation (Lisch and Bennetzen, 2011; Mirouze and Vitte, 2014; Slotkin and Martienssen, 2007). These mechanisms are difficult to investigate in *Arabidopsis thaliana*, as the plants possess few transposons and show only a low methylation level. Maize, on the other hand, has a giant genome, which is mainly composed of TEs (Schnable et al., 2009; Tenaillon et al., 2011). In this work, DNA and RNA sequencing enabled the investigation of parallel changes in the methylome and transcriptome of maize roots and revealed a different adaptation of mRNA transcripts and DNA methylation between samples indicating nutrient-specific strategies. The extent of transcriptional change was relatively similar between the two macronutrients while in -Zn more than one third fewer DEGs were found. The percentage of up- and down regulation was almost 50% in each treatment. It could be shown that many of the differentially methylated transposable elements seemed to have a gene expression regulatory effect on nearby genes in all treatments and a significant correlation was revealed for -N and -P but not for -Zn. Secco et al., 2015 found that many hypermethylated TEs were located near induced genes in rice, while other teams suggested that hypermethylation of TEs leads to decreased gene expression of nearby genes in *Arabidopsis thaliana* (Ahmed et al., 2011; Eichten et al., 2012; Hollister and Gaut, 2009). It was found in this work that most TEs were hypomethylated when compared to control and the closely located genes were both up- and down regulated in more or less equal parts.

Surprisingly, no significant correlation between differential methylation in promoters and/or gene bodies and gene expression was present in -N and -P, but only in -Zn. A majority (between 86% and 95%) of all DMRs was located in TEs, which was expected considering the genome consisting to 85% of TEs (Schnable et al., 2009; Tenaillon et al., 2011). As a consequence, only a comparably small number of DMRs was located

in genes and promoters and of these only between 6% and 29% in both symmetrical contexts were both differentially methylated and differentially expressed. Association of differential DNA methylation and highly differential expression of genes in maize was very rarely found by Li et al., 2015 as well. The percentages of genes both differentially expressed and methylated were not higher than can be expected by coincidence in -N and -P. While in -Zn a significance was lacking for the correlation between differential methylation in TEs and gene expression of close genes, a significance was found for differential methylation in genes and promoters and the gene expression. Here again, demethylation did not exclusively cause overexpression or downregulation of a gene but occurred with both cases.

5.5 Influence of small RNA amount on methylation loss

The assumption that the number of small RNAs which take part in DNA methylation, especially CHH methylation, decreases with decreasing amount of methylation did not hold true. Even though there was a high variability in the amount of small RNAs between the treatments, only -Zn really did lose a high amount of small RNAs compared to the control. The loss of methylation was restricted to the symmetrical contexts. RNA-directed DNA methylation occurs in all cytosine contexts for *de novo* methylation, but the symmetrical contexts are mainly independent of RdDM during replication (Matzke and Mosher, 2014). Therefore, the lacking loss of CHH methylation could be one explanation for a lack of parallel consequent decrease in small RNAs, because CG and CHG contexts can also lose methylation via reduced maintenance methylation or increased active demethylation. Indeed, -N and -Zn samples showed a small decrease in expression of maintenance methylation enzymes.

6 Conclusion

By analyzing the methylome and transcriptome of maize plants grown on different nutrient-deficient conditions, it could be shown that nutrient deficiencies invoked a decrease in the methylation level, but to a different extent in the different deficiency samples, indicating nutrient-specific adaptation. The assumption of a strong species- and tissue-specific adaptation is supported when comparing the results to the literature. Even though a massive change in the methylation pattern of the samples was observed, the correlation with gene expression change was small and the effect of demethylation causing higher gene expression which was found by some researchers, could not be confirmed. Still, significant correlation between differential methylation and differential gene expression was encountered for a minority of genes.

There are various reasons that might explain the inconsistencies concerning influence of hypo- and hypermethylation on gene regulation as well as the aforementioned discrepancies in the methylation level following nutrient stresses between plant species as well as between different tissues of one plant species. On the one hand, especially interspecific differences can be explained as highly species-specific adaptation strategies. Additionally, both intra- and interspecific variances might also be caused by tissue-specific mechanisms. Another reason probably is the lack of consistency in the experimental designs. Up until now, there is no consensus about how to define a DMR (e.g. how many cytosines should be regarded, how big the methylation difference needs to be, how long the DMR should be minimally/maximally, in which distance to a gene the DMR needs to be positioned and in which genome feature etc.). In addition, the duration of the stress (short-term vs. long-term) as well as the intensity (weak vs. strong) can influence the change in methylation and gene

expression. These variables and non-standard procedures can contribute to contradictory findings about DNA methylation and gene regulatory effects (Secco et al., 2017). Another fact that complicates conclusions about correlations between DNA methylation changes and gene expression is that DNA methylation is definitely not the only mechanism to influence gene expression and it is not the only epigenetic method to do so. Histone methylations and acetylations, for example, were also already shown to correlate with gene expression (Latzel et al., 2013; Tsaftaris et al., 2003; Yong-Villalobos et al., 2015). Therefore, many different factors contribute to the regulation of gene expression and influence each other and observing one piece of the puzzle at a time might obscure the whole picture. A detailed histone methylation and acetylation code, for example, in parallel with the DNA methylome, is maybe required to unravel the function in gene regulation by DNA methylation. The many different mechanisms and factors contributing to the regulation of gene expression might also provide an explanation for the divergent methylation adaptation between the different nutrients (massive methylation loss in -N and -Zn, but minor loss in -P). During the evolution of maize, the plants might have developed diverse adaptation strategies for different nutrient deficiency conditions. In the coping mechanisms for the deficiency of some nutrients, DNA methylation might play a bigger role than in coping for deficiency of other nutrients where different mechanisms might be more important or the involved mechanisms influence each other in a different way.

The high adaptability of DNA methylation, however, shows that it plays an important role (or roles) for the plant, because it is unlikely that plants would provide energy for the adaptation without purpose and benefit for them. It was many times assumed that DNA methylation is involved in gene expression and/or transposable element regulation but generalities about the role of DNA methylation in these functions are still

difficult to establish. One attempt to tackle the problem of experiments being not comparable due to strongly divergent experimental designs, is to gain a higher consensus about defining DMRs as well as to establish and apply more standardized procedures.

Though it is not yet possible to make a universally valid declaration about the influence of hypermethylation in transposable elements, gene bodies or promotors on the expression of genes that applies to all plant species, the assumption that differential methylation in TEs has an influence on nearby gene expression is further supported by the here described data.

Considering the methodology used in the experiments, RBBS has some advantages compared to whole genome bisulfite sequencing. It allows representative methylation analysis at whole genome level at reduced time and especially cost while maintaining a high sequencing depth by enriching the library for CG containing motifs and it has minimal DNA requirements (Doherty and Couldrey, 2014; Smith et al., 2009; Stockwell et al., 2014; Wang, Xia, et al., 2013; Wang et al., 2012; Wang, Liu, et al., 2013). Nevertheless, it also has its drawbacks, the main of which for the experiments of this work lies in the aggravated comparability of methylome and transcriptome data. As information about the expression level of all genes of the entire genome was present, but methylation information only for about 14% of the genome (18% of all cytosines), it was not possible to evaluate the influence of methylation on gene expression for all genes, but only for a subset. This was made even harder by the fact that, for example, some genes are covered in full length by the reduced representation genome, while others are only partly covered, making detection of a DMR in these genes more improbable. In the attempt to alleviate this difficulty in comparison a cutoff was set for genes that were only covered partly by the reduced representation genome. Still, the

results may be biased and it is possible that with reduced representation bisulfite sequencing a significant correlation between DMRs in genes, promoters or TEs and gene expression escaped the analysis. However, the experiments clearly showed that maize roots adapt their DNA methylation when grown on specific nutrient deficiencies and that methylation differences and expression are correlated for a small number of genes.

However, a direct expression regulation was most probably not the only function of DNA methylation changes due to nutrient deficiencies in this work, especially when concerning heritability. It has been suggested that stress-induced changes in DNA methylation might function as “stress-priming” for the next generation(s) (Boyko and Kovalchuk, 2011; Crisp et al., 2016; Gutzat and Mittelsten Scheid, 2012; Iwasaki and Paszkowski, 2014; Mirouze and Paszkowski, 2011). Epigenetic mechanisms involved in heritable adaptation to stress would provide a much more dynamic and faster adaptation strategy than changes in nucleotide sequence. A short-term “boost” for coping with nutrient stresses in the next generations through a changed gene expression introduced by differential methylation is improbable for the results of the here described experiments. The results did not imply heritable changes in the gene expression of nutrient-regulated genes as these were rarely differentially methylated or controlled by differential methylation in nearby transposable elements. Generally, there was only a minor effect of DNA methylation on gene expression visible. In addition, recent research also found no improved coping with deficiencies in plants that grew on limited P supplied soils for many consecutive generations. On the contrary, genetically identical plants that have been growing on well supplied soils established better under both P-deficient and P-sufficient conditions than the allegedly “deficiency-adapted” plants (Schönberger et al., 2016).

The encountered changes in the DNA methylation level might function in enhancing responsiveness of the plants due to stress exposure by a hyperinduction of transcription (Crisp et al., 2016). However, despite the loss of methylation, no overall increase in gene expression was found for any of the deficiency samples, but even a small decrease. Therefore, a more likely purpose of deficiency-induced DNA methylation changes could be a less targeted adaptation strategy. DNA methylation helps to protect the DNA from mutations to maintain nucleotide sequence. Additionally, it aids in preventing excess recombination events, especially at the centromere and pericentromeric regions, which are usually highly methylated in both plants and animals (He et al., 2011; Mirouze et al., 2012). Mutations and crossovers increase the genetic diversity of organisms and thereby augment the chance for the development of beneficial traits in the phenotype. Loss of DNA methylation was found to come along with an increase in the frequency of recombination events and mutations as well as the movement of transposable elements in the genome (Boyko and Kovalchuk, 2011; Mirouze et al., 2009, 2012; Mirouze and Paszkowski, 2011; Mlura et al., 2001; Reinders et al., 2009; Tsukahara et al., 2009). Thereby the genetic diversity would be increased in the next generation leading to new features and possible establishment of new phenotypes even without a long-term memory of the epigenetic status in following generations. It was suggested that the increase in genetic variability might not even be completely random but that changed frequency in recombination events could be directed to a certain extent to specific genomic regions (Boyko and Kovalchuk, 2011). A higher genetic diversity and new traits could be a great advantage especially on population level, increasing the chance of adaptation to adverse conditions in the population (Boyko and Kovalchuk, 2011). Such a mechanism could be regarded as an evolutionary driver promoted by plants under stress conditions.

This assumption introduces the question why the methylation loss in -P samples was weaker than in the other two samples. On the one hand, stress in plants also leads to defects or difficulties in maintaining proper functioning of plant-intern mechanisms, so that a less pronounced downregulation of methylation in -P samples might be due to defective regulation of expression of particular genes. For example, the slight upregulation of enzymes for maintenance methylation in -P samples in contrast to the downregulation in -Zn and -N samples might be due to phosphorus deficiency-caused regulation difficulties resulting in “unintended” reduced methylation loss. Another explanation might be that the process of demethylation was slower or started later in -P samples than in the other two samples so that a stronger methylation loss would have been encountered as well in -P samples at a later time point. Beside the duration of stresses, also the intensity of stress conditions could be a factor for differential DNA methylation adaptation (Boyko and Kovalchuk, 2011). The active promotion of mutations and crossover events is a somewhat drastic measure as they can also lead to disadvantageous outcomes for the next generation. Especially crossovers in centromeric or pericentromeric regions carry the risk of leading to improper chromosome segregations and aneuploidy. Even though plants are usually less affected by aneuploidy than mammals, the frequency and location of crossover events is highly controlled (Siegel and Amon, 2012). Assuming that an intense stress level is needed in plants to trigger such measures would mean that the stress level in -P samples maybe was not high enough to induce strong methylation loss, thereby implying that the epigenetic response to the nutrient stresses is after all a more general stress response depending on the intensity of the stress rather than a specific nutrient. On the other hand, other (epigenetic) factors are probably involved in changes in recombination frequency as well (Boyko and Kovalchuk, 2011) so that the

rearrangement of genome stability might be caused to a different extent by different factors in -P and the two other deficiency samples. Finally, methylation level changes and accompanying genome stability changes seem to be dependent on the kind of stress present as for example a strong loss of DNA methylation was found due to salt stress in maize (Steward et al., 2002) and due to cold stress in *Antirrhinum majus* (Hashida et al., 2006). Likewise, hypomethylation also resulted from heavy metal stress in hemp and clover (Aina et al., 2004), while irradiation stress in *Arabidopsis* (Kovalchuk et al., 2003) and water deficit in pea (Labra M. et al., 2002) led to hypermethylation. Radiation, for example, is known to cause tremendous destabilization of genomes (point mutations, double strand breaks, movement of TEs) which can lead to strong damage and disturbed growth and development of the plants. The hypermethylation under radiation stress can thereby protect from excessive instability of the genome (Kovalchuk et al., 2003). In other stresses, on the other hand, increased genome instability might lead to the establishment of favorable new traits. Considering that different stresses invoke different methylation responses leads to the suggestion that different nutrient deficiencies might also induce variable changes in the methylation level, leading to a “fine-tuning” of genome rearrangements and gene expression.

6.1 Outlook

To tackle the inconsistencies concerning the influence of DNA methylation on gene expression in plants, further research is needed. In addition to higher standardization and consensus about procedures, future experiments should also include the observation of the interplay between different epigenetic mechanisms, e.g. looking at histone and DNA modifications at the same time. Additionally, samples should be

harvested after short-term, medium-term and long-term stress to analyze how DNA methylation changes behave over time. To confirm or negate species-, tissue- and nutrient-specific DNA methylation adaptations, comparisons between plants, tissues and further nutrients are needed. For investigation of heritability of gene expression changes due to DNA methylation adaptation, subsequent plant generations should be involved where possible.

Open questions also remain for the assumption that DNA methylation adaptation due to (nutrient) stresses is involved in rearranged or more frequent recombination events, mutations and movement of transposable elements. Comparisons of DNA methylation changes due to different kinds of stresses (nutrient stress, cold stress, radiation stress, pathogen-induced stresses, drought and water stress etc.) as well as different intensities and durations of these stresses are important to get to know which conditions induce genome stability changes and in which form (increase, decrease or relocation of genome stability). Concerning the heritability of such changes different findings have been made so far, including maintenance of these changes only in the next one or two generations (Boyko et al., 2010; Kathiria et al., 2010) or prolonged in at least the next four generations (Molinier et al., 2006). Consequently, further research regarding heritability of induced genome stability changes, e.g. by applying one time only or repeated exposures to stress in subsequent generations and testing of possible impacts on heritability, is significant for gaining a more differentiated picture about if and how heritable genome stability changes are brought about.

6.2 Summary

Though there are still a lot of open questions and things to learn about the role of DNA methylation in plants, this work provides evidence that epigenetic mechanisms influence gene expression, but that this is not the only function and maybe not even the most important one, at least when concerning heritability. To our knowledge, the impact of the deficiency of three different essential nutrients on the methylome and transcriptome of maize plants has not been compared before and provided a valuable basis for further research to overcome the gap between model plants like *Arabidopsis*, on which most experiments are still done, and plants which play a tremendous role in agriculture. The results suggested a role of DNA methylation adaptation in the increase of genetic diversity for following generations and by this the functioning as evolutionary driver. All in all, this work provides basic research able to inspire further investigations about the functions of DNA methylation, especially with respect to (nutrient) stress adaptation strategies, which can contribute to valuable knowledge applicable in plant breeding and crop protection as well as in evolutionary studies.

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	Publications
2018	Mager, S. and Ludewig, U. (2018), ”Massive Loss of DNA methylation in nitrogen-, but not in phosphorus-deficient Zea mays roots is poorly correlated with gene expression differences”, *Frontiers in Plant Science*, Vol. 9 No. April, pp.1-14
2016	Schönberger, B., Chen, X., Mager, S. and Ludewig, U. (2016), ”Site-dependent differences in DNA methylation and their impact on plant establishment and phosphorus nutrition in Populus trichocarpa”, *PLoS ONE*, Vol. 11 No.12, available at: https://doi.org/10.1371/journal.pone.0168623.
In Preparation	Mager S, Ludewig U, Schönberger B. ”Zinc deficiency in maize causes expression change of Zn-regulated genes and strong loss of DNA methylation”
	Further Experience
10/2015 - today	**Volunteer work Nature and Biodiversity Conservation Union (NABU)** Plant and amphibians mapping, biotope maintenance Data entering and maintenance with qGIS Organization of NABU representations at public/internal events
03/2018	**Seminar “Rhetoric for lecturers - The art of speech, convincement and presentation”**, Hochschuldidaktikzentrum Universitäten Baden-Württemberg
02/2018	**Seminar “Negotiation training for volunteers in nature protection”**, NABU Baden-Württemberg
02/2018	**Seminar “Sonorous speaking: Voice training for lecturers”**, Hochschuldidaktikzentrum Universitäten Baden-Württemberg
01/2018	**Seminar “Practical rhetoric: How to give competent talks and presentations”**, University of Hohenheim
12/2017	**Seminar “Authentic und constructive communication”**, University of Hohenheim
07/2017	**Workshop “Agriculture for blooming landscapes”**, NABU Summer Academy, Bad Boll
05/2017	**Seminar “Plant Mapping - theory and practice”**, NABU, Ostfildern
07/2016	**Workshop „Natura 2000”**, NABU Summer Academy, Bad Boll
10/2013 - 04/2014	**Volunteer work Biological Station** Bonn/Rhein-Erft Project: “‘RegioSaatGut’ - Preservation and resettlement of regionally adapted flowering plants“
10/2004	**Student Exchange USA** with North Andover High School, Massachusetts

Conference/Seminar Participation

04/2018	**Presentation "Epigenetic Adaptation: Nutritional Regulation of DNA Methylation and Gene Expression in Maize"**, seminar series "Biological Signals", University of Hohenheim
09/2017	**'Poster Attraction Talk'** and poster presentation, Botanikertagung 2017
2014 - 2017	Various poster presentations

References

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Memberships

Since 2018	The Ecological Society of Germany, Austria and Switzerland (GfÖ)
Since 2017	German Society for Plant Sciences (DBG)
Since 2015	Nature and Biodiversity Conservation Union (NABU)

Recreational Activities

Botany, Plant Science
Environment and nature protection
Agriculture
Hiking, Psychology

Ostfildern, April 2018