The epigenetic regulation of C4-photosynthesis genes from *Zea mays* heterologously expressed in *Oryza sativa indica* in response to different stimuli

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Abstract

The critical nutritional situation presents the world population with great problems. In order to meet the requirements posed by this situation, a minimum of 60 % increase in agricultural productivity until 2050 is an indispensable necessity. Common breeding approaches lack the efficiency to achieve the desired increase in crop yields. Therefore, transgenic or genome editing approaches might offer a possible solution for the critical nutritional situation by providing high-yielding plants that outperform conventional crops. At numerous locations, species performing C4-photosynthesis have proven to be superior to C3-species. Therefore, the introduction of the C4-mechanism into C3-crops might increase their productivity. This is due to the ability of C4-plants, to avoid the necessity of photorespiration. In order to realize a functional C4-mechanism in C3-plants, the correct regulation of gene expression has to be ensured. In this context, the question arises, whether the regulators responsible for C4-gene expression would have to be introduced into C3-crops, too. To answer this question, the regulation of C4-genes in transgenic rice has been evaluated in the present thesis on the mRNA level and on the chromatin level. In this context, light inducibility of the two key genes C4-*PEPC* and C4-*PPDK* has been tested and revealed a regulation of the respective mRNA levels in transgenic rice which corresponded to the regulation of the mRNA level in the actual C4-plant maize. It was accompanied by histone modification profiles which were also very similar to the histone modification patterns in maize. In addition to the ability to adapt C4-*PEPC* and C4-*PPDK* expression to illumination, the occurrence of a diurnal rhythm has been tested for C4-*PEPC* and the associated histone modifications. Again, transgenic rice demonstrated a regulation of C4-*PEPC* that corresponded to the one in maize and demonstrated a similar behavior with regard to the time-course. Like mRNA levels, histone modifications also mirrored events in the donor species maize. Finally, the influence of nitrogen availability was tested as an example for a metabolic stimulus. In this context, the mRNA levels in transgenic rice demonstrated a stronger reduction in response to nitrogen depletion, than the mRNA levels in maize. Apart from this, the reaction of acetylation levels at the proximal promoter of C4-*PEPC* differed in the two species, as it was not influenced by nitrogen depletion in maize, but reduced in the same situation in transgenic rice. Altogether, the results indicated that the C3-crop rice seems to be able to regulate the expression of C4-genes in a similar way to the regulation in actual C4-plants.

Keywords: Epigenetic regulation, chromatin, C4-photosynthesis

Zusammenfassung

Die kritische globale Ernährungssituation stellt die Weltbevölkerung vor große Probleme. Um den durch diese Situation gestellten Anforderungen begegnen zu können, ist eine mindestens 60 prozentige Steigerung der landwirtschaftlichen Erträge bis zum Jahre 2050 notwendig. Herkömmlichen Zuchtmethoden fehlt es an Effizienz, um diese Ertragssteigerung herbeizuführen. Gentechnische oder Genome Editing Ansätze könnten eine mögliche Lösung für die Problematik darstellen, da hierdurch Pflanzen generiert werden könnten, deren Erträge diejenigen herkömmlicher Getreidesorten übertreffen. An zahlreichen Standorten sind C4-Pflanzenarten C3-Arten überlegen. Aus diesem Grund könnte das Etablieren des C4-Mechanismus in C3-Arten deren Produktivität erhöhen. Grund hierfür ist das Umgehen der Photorespiration. Um einen funktionierenden C4-Mechanismus in C3-Pflanzenarten zu gewährleisten, muss die korrekte Regulation der Genexpression sichergestellt sein. In diesem Zusammenhang stellt sich die Frage, ob die Regulatoren, die für die C4-Genexpression verantwortlich sind, ebenfalls in C3-Pflanzen eingebracht werden müssten. Um diese Frage zu beantworten, wurde die Regulation von C4-Genen in transgenem Reis auf der mRNA- und der Chromatin-Ebene evaluiert. In diesem Zusammenhang wurde die Lichtinduzierbarkeit der C4-Schlüsselgene C4-*PEPC* und C4-*PPDK* untersucht. Hierbei konnte eine Regulation des entsprechenden mRNA-Levels in transgenem Reis beobachtet werden, die der Regulation des mRNA-Levels in der C4-Art Mais glich. Dieses trat in Begleitung eines ähnlichen Histonmodifikationsprofils auf, wie es auch in der C4-Pflanze Mais beobachtet werden kann. Zusätzlich zur Fähigkeit C4-*PEPC* und C4-*PPDK* an die Belichtungssituation anzupassen, wurde das Auftreten eines diurnalen Rhythmus für C4-*PEPC* mRNA und die assoziierten Histonmodifikationen verifiziert. Wie auch das mRNA-Level, so spiegelten die Histonmodifikationen die Prozesse in Mais wider und zeigen ein ähnliches Verhalten im Zeitverlauf. Abschließend wurde der Einfluss von Stickstoffverfügbarkeit als metabolischer Stimulus getestet. Hierbei konnte unter Stickstoffmangel eine stärkere Reduktion des mRNA-Gehalts in transgenem Reis im Vergleich zur Reduktion des mRNA-Gehalts in Mais beobachtet werden. Zudem zeigte das Acetylierungslevels am proximalen Promoter von C4-*PEPC* Unterschiede, da es in Mais durch Stickstoffmangel nicht beeinflusst wird, in Reis jedoch eine Reduktion zeigt. Zusammenfassend zeigen die vorliegenden Ergebnisse, dass die C3-Pflanze Reis dazu in der Lage ist, die Expression von C4-Genen in ähnlicher Weise zu regulieren, wie C4-Pflanzen dies tun.

Schlagworte: Epigenetische Regulation, Chromatin, C4-Photosynthese

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List of abbreviations

2PG	2-Phosphoglycolate
3PGA	3-Phosphoglycerate
ATP	Adenosine triphosphate
Bp	Basepairs
CA	Carbonic anhydrase
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOG	Deoxyglucose
EDTA	Ethylenediaminetetraacetic acid
EMBL-EBI	The European Bioinformatics Institute
Fw	Forward
GDC	Glycine decarboxylase
GGT	Glutamate Glyoxylate aminotransferase
GLYK	Glycerate 3-kinase
GOX	Glycolate oxidase
GXR1	Cytosolic glyoxylate reductase
GXR2	Plastidial glyoxylate reductase
H3C	Invariant C-terminal end of H3
H3K18ac	Acetylated lysine 18 of histone H3
H3K4me3	Trimethylated lysine 4 of histone H3
H3K9ac	Acetylated lysine 9 of histone H3
H4K5ac	Acetylated lysine 5 of histone H4
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HKMTs	Histone lysine methyltransferases
hnRNA	heterogenous nuclear RNA
HPR1	NADH-dependent hydroxypyruvate reductase
IRRI	International Rice Research Institute
ME	Malic enzyme
MeCP2	Methyl CpG binding protein 2
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OXA	Oxaloacetate
PCR	Polymerase chain reaction
PEP	Phosphoenol pyruvate

PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PMSF	Phenylmethylsulfonylfluoride
PPDK	Pyruvate orthophosphate dikinase
qPCR	Quantitative Real-time PCR
rev	Reverse
RNA	Ribonucleic acid
R-SUPs	Regulated secondary upstream peaks
RT	Reverse Transcriptase
RUBISCO	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SAM	S-adenosylmethionine
SGT	Serine:Glyoxylate aminotransferase
SHMT	Serine hydromethyltransferase
TE	Transposable element
TEA	Tris-acetate-EDTA buffer
TIS	Transcription initiation site
TSA	Trichostatin A
VE-H_2O	Demineralized water

1 Introduction

1.1 The nutritional situation in the light of environmental changes and population growth

In order to meet the challenges imposed by the growing population in developing countries, an increase in global agricultural productivity is unavoidable. The nutritional situation is presenting severe problems already today. For example in 2010, 925 million people were estimated to be exposed to hunger and malnutrition (Kajala *et al.*, 2011). This situation will further be affected by the progressing climate change, accompanied by changing weather conditions, leading to water scarcity and droughts (Hibberd, Sheehy and Langdale, 2008) and is combined with a strong worldwide population growth, resulting in 9 billion people in 2050 (Kajala *et al.*, 2011). Rice as agricultural crop plays a crucial role in this context, as it provides the basic food for a large part of the population, especially in developing countries. In 2008, 27 people were nourished by 1 hectare of rice producing land. Due to the increase in population, accompanied by a decrease in cultivation areas, the productivity of each hectare will have to be raised. By 2050, 43 people will have to be fed by the same area that was nourishing 27 people in 2008. This means that one aim of current efforts to increase agricultural productivity is a minimum of 60 % increase in rice productivity until 2050. Therefore, while in 2007 250 million tons of carbon where fixed in rice grains, the amount of fixed carbon will have to be elevated to 400 million tons by the year 2050 (Sheehy and Ferrer, 2008; Kajala *et al.*, 2011). Common breeding approaches pursued today will scarcely result in an increase in crop yields, strong enough to meet the requirements that will have to be met in the future (Kajala *et al.*, 2011). Additionally, the Green Revolution, achieved by the development of fertilizer responsive, efficient rice cultivars by the International Rice Research Institute (IRRI), which took place in the 1960s, has already been meeting its barriers in 2008. As described by Kropff *et al.* (1994) and Sheehy and Ferrer (2008), its influence on rice production already slowed down in the 1980s, as there was no further increase in the average yield of rice in tons per hectare and per year. Instead, changes in rice yields decreased again (Kropff *et al.*, 1994; Sheehy and Ferrer, 2008).

In this context, it is of great value that numerous C4-species already demonstrated means to acquire 50 % higher radiation use efficiencies in comparison to C3-plants like rice (Kiniry *et al.*, 1989; Mitchell and Sheehy, 2006; Hibberd, Sheehy and Langdale, 2008). This trait is additionally accompanied by an ameliorated proficiency in the use of water and nitrogen, rendering C4-crops even more efficient

compared to C3-plants (Sage and Pearcy, 1987; Kiniry *et al.*, 1989; Makino *et al.*, 2003; Sage, 2004; Mitchell and Sheehy, 2006; Hibberd, Sheehy and Langdale, 2008). This is of special significance with regard to the changing environmental conditions. In the light of these information, approaches to introduce the C4-mechanism into C3-crops, with special regard to rice, seem most valuable and future-orientated. They might offer a possible solution for already existing nutritional problems.

1.2 Photosynthesis

The means by which biomass providing the foundation of nourishment is produced is photosynthesis. In photoautotrophic organisms like plants, the photosynthetic mechanism secures the energy supply by the fixation of carbon (Koussevitzki *et al.*, 2007) with the help of electromagnetic energy provided by sunlight. The process can be divided in two distinct parts. First of all the light reaction, which is characterized by the oxidation of water and the transport of electrons by a series of enzymes, to produce adenosine triphosphate (ATP) and reduction equivalents. Subsequently, carbon dioxide (CO_2) is reduced and fixed in form of carbohydrates in the dark reaction. The basic mechanism of the dark reaction is the Calvin cycle, which is also known as the pentose phosphate pathway. It consists of three distinct reactions. A carboxylation of ribulose-1,5-bisphosphate, which acts as acceptor molecule, is performed by the enzyme ribulose-1,5-bisphosphate carboxylase. This leads to the formation of two molecules of 3-phosphoglycerate (Gowik and Westhoff, 2011). 3-phosphoglycerate is reduced to glyceraldehyde 3-phosphate, which is used for the synthesis of more complex carbohydrates. Finally, ribulose-1,5-bisphosphate is regenerated. Concerning the Calvin Cycle, the plant meets a serious problem based on the fact that the key enzyme, ribulose-1,5-bisphosphate-carboxylase (RUBISCO), which is responsible for the transfer of carbon to the acceptor of the first reaction step, is prone to performing oxygenation reactions if the temperature and surrounding CO_2 concentrations are suboptimal. In this case, it is not CO_2, which binds to the active centre of the enzyme, but instead O_2 (Brooks and Farquhar, 1985). This activity leads to the formation of 2-phosphoglycolate (2PG) instead of a second molecule of 3-phosphoglycerate (3PGA). As 2PG cannot be utilized during the Calvin Cycle (Peterhansel *et al.*, 2010) and as it is also toxic for the plant (Anderson, 1971), it has to be converted to 3PGA in peroxisomes, mitochondria and chloroplasts. At the end of this mechanism, 3PGA is generated (Bauwe, Hagemann and Fernie, 2010). This unavoidable process is referred to as photorespiration, which is also known as the oxidative C_2 cycle and is accompanied by a loss of energy for the plant (Peterhansel *et al.*, 2010). Under agreeable conditions, the described effect is not necessarily problematic. However, increasing ambient air

temperatures force the plant to close its stomata. This is a necessary prerequisite to avoid water loss. The closure of the plants stomata however can lead to a suboptimal ratio of CO_2 and O_2, thus provoking photorespiration caused by RUBISCOs oxygenase activity. Due to the recycling of 2PG, the oxygenase activity of RUBISCO leads to a net reduction of photosynthesis not only by the inhibition of direct CO_2 fixation (Chollet, 1977). Apart from this effect, the necessary detoxification of 2PG, leads to high carbon and energy losses (Bauwe, Hagemann and Fernie, 2010). In moderate conditions, these losses can reach about 20 % of a C3-plants net-photosynthesis, an amount that is further increased under warm and dry conditions (Cegelski and Schaefer, 2006). The process of photorespiration requires the activity of a series of different enzymes located in different cellular organelles. The toxic compound 2PG is hydrolyzed by a 2PG phosphatase. This reaction takes place in the chloroplast and results in the formation of glycolate (Bauwe, Hagemann and Fernie, 2010). Glycolate leaves the chloroplast and enters the peroxisome (Reumann and Weber, 2006). Here, the enzyme Glycolate Oxidase (GOX) catalyzes a reaction in which glyoxylate and H_2O_2 are generated from glycolate and molecular oxygen (Bauwe, Hagemann and Fernie, 2010). During the next step of the mechanism, the transamination of glyoxylate leads to the formation of glycine. This is achieved by the action of two enzymes in parallel, the Serine:Glyoxylate Aminotransferase (SGT) which uses serine as amino donor, and the Glutamate Glyoxylate Aminotransferase (GGT) which uses either glutamate or alanine as amino donor (Liepman and Olsen, 2001; Bauwe, Hagemann and Fernie, 2010). Under stress, glyoxylate can escape this transamination process. In this case, it becomes a substrate for the cytosolic glyoxylate reductase GXR1, or the plastidial glyoxylate reductase GXR2 (Allan *et al.*, 2009). In the mitochondria, the enzymes glycine Decarboxylase (GDC) and serine hydromethyltransferase (SHMT) use two glycines to produce one serine (Bauwe, Hagemann and Fernie, 2010) which moves into the peroxisome. Its amino group is transferred to glyoxylate by the aforementioned activity of SGT. This results in the generation of hydroxypyruvate which becomes a substrate for the NADH-dependent hydroxypyruvate reductase (HPR1). In a final step, which takes place in the chloroplast, 3PGA is regenerated by the action of glycerate 3-kinase (GLYK). Every time this cycle is completed, one of the four carbon atoms that entered the process in the form of 2PG is lost, while three enter the Calvin Cycle. In this context, photorespiratory CO_2 is generated. At the end of the process, 2 molecules of 2PG can be used to recover one molecule of 3PGA (Bauwe, Hagemann and Fernie, 2010). Apart from this photorespiration also leads to a loss of nitrogen (Reumann and Weber, 2006). To sum up, the oxygenase activity of RUBISCO can lead to high carbon and energy losses in C3-plants, especially, when environmental conditions are suboptimal and lead to increased O_2 concentrations in the cells. The described process can result in an oxidation of up to 50 % of the photosynthetic products in C3-plants (Kennedy, 1976), an effect which is strongly reduced in C4-plants (Zelitch, 1975). Already in 1976, a strongly reduced photorespiration rate could be

demonstrated for tissue cultures of C4-plants in comparison to tissue cultures of C3-plants. In tissue cultures generated from C4-plants, the observed photorespiration rate accounted for only one-half to one-third of the photorespiration rate observed in tissue cultures derived from C3-plants (Kennedy, 1976).

1.3 The evolution of C4-photosynthesis

The loss of CO_2 and energy caused by the oxygenase activity of RUBISCO has led to the evolution of a series of CO_2 concentrating mechanisms in plants (Sheen, 1999), to avoid the necessity of photorespiration. One of these is C4-photosynthesis which is performed for example by *Zea mays.* The basis for the development of a functional C4-mechanism was the use of already existing enzymes of C3-plants in a new context. These modifications of the already existing biochemical processes led to a strong increase of photosynthetic efficiency. Considering that C4-plants only account for a low percentage of under 4 % of all land plant species (Sage, Monson and Li, 1999; Sage, Wedin and Li, 1999), the fact that they are responsible for 25 % of the planets primary productivity (Sage, 2004) underlines their efficiency in comparison to C3-plants. Due to the increase in photosynthetic efficiency and the resulting advantages in comparison to C3-plants especially at arid and dry locations, it does not surprise that C4-photosynthesis convergently evolved in independent plant lineages, altogether more than 50 times (Sage, 2004; Christin, Salamin, *et al.*, 2008; Peterhansel, 2011). C4-photosynthesis evolved mainly in different grass species and sedges but also in dicotyledonous plants, like in the Amaranthacean family (Wang *et al.*, 2009; Peterhansel, 2011). It is supposed that C4-photosynthesis evolved for the first time in grass species during the Oligocene, 24 to 35 million years ago (Christin *et al.*, 2008; Vicentini *et al.*, 2008). This time period corresponds to a strong CO_2 decline 32-35 million years ago (Sage, 2001; Pagani *et al.*, 2005; Osborne and Beerling, 2006; Tipple and Pagani, 2007). Christin *et al.* (2008) reported an increased probability for the evolution of C4-photosynthesis for the last 27 million years, which is also the period in which CO_2 levels showed a lower concentration than 500 ppm (Pagani *et al.*, 2005).

The fact that no other polyphyletic feature demonstrates a comparable amount of independent evolutionary events underlines the importance of C4-photosynthesis for the evolution of land plants. It is, therefore, not surprising that, considering the fast growing population and environmental changes, the idea has been developed, to introduce the C4-mechanism into important C3-crop species like rice.

1.4 The biochemistry of C4-photosynthesis

A necessary prerequisite and one of the most characteristic features of C4-photosynthesis is the participation of two distinct cell-types in the photosynthetic mechanism. Mesophyll cells and bundle sheath cells each harbor a specific set of enzymes determining their role in C4-photosynthesis (Sheen and Bogorad, 1987; Langdale *et al.*, 1988; Nelson and Langdale, 1989). The aim of this separation is to fix CO_2 in the mesophyll cells and to transport it to the bundle sheath cells, where RUBISCO is located, to create increased CO_2 concentrations which present an optimal atmosphere for the carboxylase activity of the enzyme (Gowik and Westhoff, 2011). This strategy prevents the oxygenase activity of RUBISCO, thus reducing the necessity of photorespiration. Mesophyll and bundle sheath cells further show a special distribution in the leaf, which is a prerequisite for a functional C4-mechanism. This feature was described as ´Kranz anatomy´ by Haberlandt in 1914, who first identified two distinct cell types in the leaves of specific grass species (Haberlandt, 1914). This special kind of leaf morphology is defined by mesophyll cells in the outer part of the leaf and at the surface, surrounding bundle sheath cells in the inner part of the leaf. While the mesophyll cells are in "contact with the intracellular airspace". The bundle sheath cells are located around the vains (Dengler and Nelson, 1999). The general structure of a leaf with Kranz anatomy is presented in (Figure 1.1).

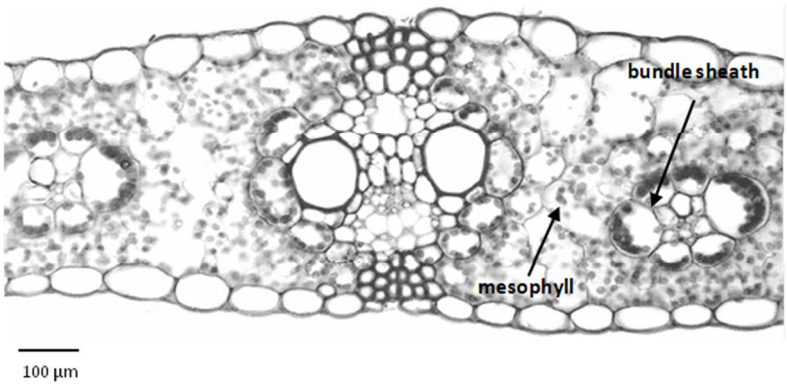

Figure 1.1: General distribution of mesophyll and bundle sheath cells in C4-plants. Bundle sheath cells are located at the inner part of the leaf, surrounding the vains. Mesophyll cells on the other hand form a wreath like structure around the bundle sheath cells and are in contact to the leaf surface and the intracellular airspace. Arrows indicate areas of mesophyll cells and bundle sheath cells. The figure is taken from Hahnen (2004) and adapted.

As a consequence energy losses due to the conversion of 2-phosphoglycolate to 3-phosphoglycerate are prevented. The basis underlying this principle is the primary fixation of CO_2 in the mesophyll cells. When CO_2 enters these cells through the stomata it is converted to hydrogen carbonate (HCO_3^-) by the enzyme carbonic anhydrase (CA). HCO_3^- is a substrate of the enzyme phosphoenolpyruvate

carboxylase (PEPC), which then performs a first fixation by transferring CO_2 to the acceptor molecule phosphoenol pyruvate (PEP). The product of this fixation is oxaloacetate (OXA). OXA is a C4-body and, therefore, responsible for the term ´C4-photosynthesis´. This term underlines one of the differences to the C3-mechanism of photosynthesis, in which the product of CO_2 fixation is the C3-body 3-phosphoglycerate. OXA enters the chloroplasts of the mesophyll cell and is subsequently converted to malate by the NADP-specific malate dehydrogenase, or to aspartate by the enzyme aspartate aminotransferase, alternatively. Malate enters the bundle sheath cells through plasmodesmata. The bundle sheath cells are protected by a permeability barrier that prevents the loss of CO_2 by diffusion out of the cells. Here, it is decarboxylated by Malic Enzyme (ME) which leads to a release of CO_2 in the target cell, thus increasing the CO_2 levels in said cell and providing an optimal CO_2 saturation for the carboxylase activity of RUBISCO. A refixation of the released CO_2 is performed by RUBISCO and the fixed carbon enters the photosynthetic carbon reduction cycle to be finally used for sucrose and starch formation. The remaining pyruvate leaves the bundle sheath cells and returns to the mesophyll cells. After phosphorylation of pyruvate by the enzyme pyruvate orthophosphate dikinase (PPDK), PEP is recovered (Langdale, 2011). Thus, the circle is closed by providing the acceptor molecule for the primary fixation of inorganic carbon provided by CO_2 (Dai, Ku and Edwards, 1993; Kanai and Edwards, 1999). The mechanism of C4-photosynthesis in *Zea mays* is schematically presented in (Figure 1.2).

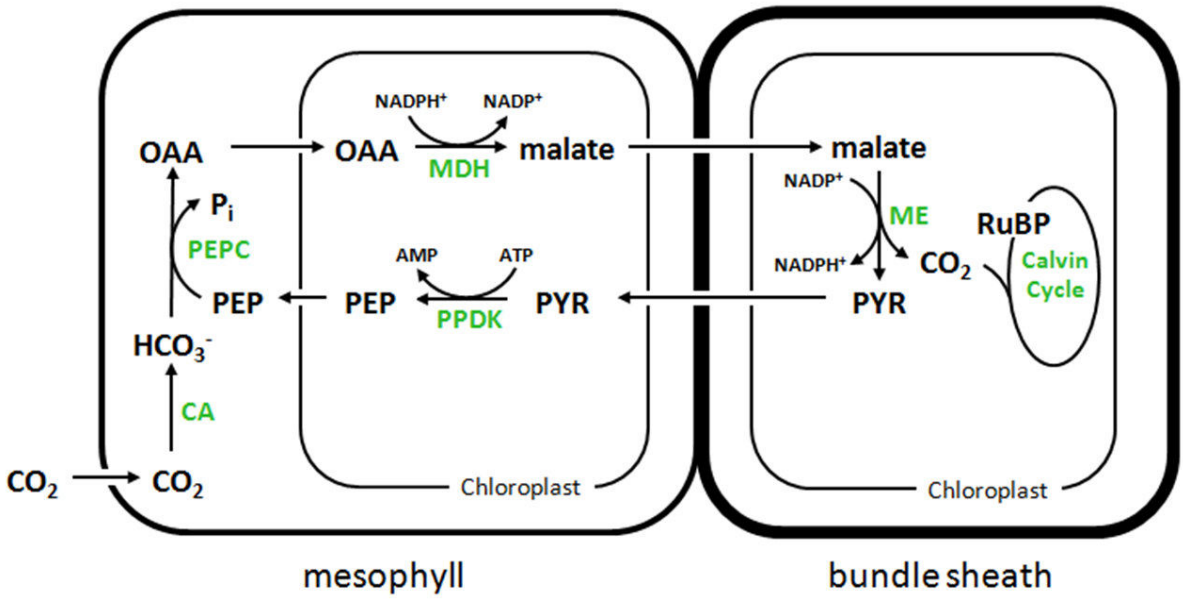

Figure 1.2: Overview of NAPD-ME type like C_4 photosynthesis. As explained in 1.3, bundle sheath cells are characterized by a diffusion barrier that prevents CO_2 from leaving the cell by diffusion. This is indicated in the present figure by a thicker cell wall. CA: Carboanhydrase; PEPC: Phospho*enol*pyruvate carboxylase; MDH: Malate dehydrogenase; ME: Malic enzyme; RUBISCO: Ribulose,1-5,bisphosphate carboxylase-oxygenase; PPDK: Pyruvate-P_i-Dikinase; HCO_3^-: Bicarbonate; PEP: Phosphoenolpyruvate; OAA: Oxaloacetate; PYR: Pyruvate; RuBP: Ribulose,1-5,bisphosphate; P-glycerate: 3-phosphoglycerate; NAPDH: Nicotinamide adenine dinucleotide phosphate (reduced); $NAPD^+$: Nicotinamide adenine dinucleotide phosphate (oxidized); AMP: Adenosine monophosphate; ATP: Adenosine triphosphate. The figure is taken from Hahnen (2004).

In the context of C4-photosynthesis, it has to be noted that the release of CO_2 in the bundle sheath cells can be catalyzed by different enzymes. Therefore, it is possible to divide plants performing C4-photosynthesis in three distinct subgroups. Those are the NADP-ME type species and furthermore plants that instead use NAD-ME or the enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Kanai and Edwards, 1999).

However, even if the C4-mechanism can overcome the disadvantages of photorespiration in C3-plants, it has to be considered that the specific features characteristic for C4-photosynthesis are associated with a higher energy requirement than it can be observed in C3-plants without photorespiration (Ehleringer and Björkman, 1977). If photorespiration is absent and does, therefore, not impair carbon fixation in the regular mechanism of C3-photosynthesis, the energy requirement of C3-plants would be 3 molecules of ATP and 2 molecules of NADPH for each molecule of CO_2 that is fixed by the plant (Kanai and Edwards, 1999). Concerning the processes that take place in bundle sheath cells of C4-plants, the same energy requirements are observed for the secondary fixation of CO_2. This is due to the fact that there are only very low levels of oxygenase activity of RUBISCO in bundle sheath cells in light (Kanai and Edwards, 1999). Nevertheless, the distribution of C3- and C4-plants differs and depends on the habitat, which could not be explained, if the C3- and the C4-mechanism would be equally efficient in general. For example, it can be observed that C4-plants show an infrequent distribution in cooler climates. Even in temperate climates, they are rarely met (Teeri and Stowe, 1976). The explanation for this phenomenon is that in fact, the C4-mechanism is in general characterized by a higher energy requirement than C3-photosynthesis. This is mainly due to the extra energy which has to be provided for the regeneration of PEP from pyruvate by the action of PPDK. This process, which is necessary to recover the primary CO_2 receptor in mesophyll cells, leads to additional energy costs of 2 molecules of ATP for each molecule of CO_2 (Kanai and Edwards, 1999). Apart from this additional energy consuming step of C4-photosynthesis, the transport of CO_2 from the mesophyll cells to the bundle sheath does not take place without losses. Actually, a leakage of CO_2 from bundle sheath cells further increases the energy costs of C4-photosynthesis *in vivo* (Henderson, von Caemmerer and Farqhuar, 1992; Hatch, Agostino and Jenkins, 1995; He and Edwards, 1996; Von Caemmerer and Furbank, 2003). In different studies, the percentage of this leakage was determined to range between 8 % and 50 %. If the frequency is estimated to account for 20 %, there would be an additional expense of 2.5 molecules of ATP for each CO_2 fixed (Kanai and Edwards, 1999). Apart from this, it has to be considered that photorespiration can also be observed in bundle sheath cells (even if only to a very small degree) and is not completely suppressed. Altogether, C4-species showing the NADP-ME type of C4-photosythesis have an energy requirement of 5.7 molecules of ATP and 2.2 molecules of NADPH per CO_2 fixed (Kanai and Edwards, 1999). As already mentioned above, C3-plants require 3 molecules of ATP and 2 molecules of NADPH. This

energy requirement however increases when photorespiration becomes a crucial aspect in C3-plants under unfavorable conditions. Under low CO_2 concentrations, the energy costs of C3 photosynthesis increase due to the necessity of photorespiration. Eventually, they exceed the additional energy costs associated with C4-photosynthesis (Sage, Wedin and Li, 1999; Edwards *et al.*, 2001), thus establishing a selection disadvantage in comparison to C4-plants under specific environmental conditions. Against the background of the higher energy requirements of C4-plants, which only allow advantages under conditions favoring a low CO_2 concentration in the cells, it does not surprise that C4-plants are superior to C3-plants especially under high temperatures and water scarcity (Björkman, Mooney and Ehleringer, 1975; Ehleringer and Björkman, 1977). High temperatures, low atmospheric CO_2 concentrations and restricted stomatal aperture favor low intercellular CO_2 concentrations, thus leading to photorespiration (Sage, Wedin and Li, 1999). The environmental conditions that correspond to these aspects can for example be found in subtropical regions like grasslands and deserts (Ehleringer and Björkman, 1977). Different models for the ongoing climatic change predict increasing temperatures and a northward shift of climatic regions. This development will lead to an increase in arid regions in developing countries (Fischer *et al.*, 2005). Therefore, crops will be exposed to increased heat-stress and drought, for example in semi-arid regions (Stewart, 2015). It has already been demonstrated that C4-plants respond in a more positive way to increasing warmth than C3-plants (Jia *et al.*, 2016). They are furthermore known to show a high drought- tolerance (Yamori, Hikosaka and Way, 2014; Jia *et al.*, 2016; Lara and Andreo, 2016). Therefore, the climatic changes, which are supposed to worsen the agricultural situation in many countries, are also characterized by conditions that favor the growth of C4-plants. Consequently, global warming might lead to a change in the distribution of C4- and C3-plants (Zhang *et al.*, 2014). It does, therefore, not surprise that in the light of increasing temperatures C4-species already today show a spread towards more northern regions in Mongolian grasslands (Wittmer *et al.*, 2010; Zhang *et al.*, 2014). This underlines the high value of efforts to introduce the C4-mechanism into C3-crops like rice which might otherwise experience severe disadvantages due to the changing climatic conditions. The ability to perform C4-photosynthesis will enable the C3-plant rice to adapt to the changing environmental conditions, thus providing higher yields to nourish an increasing world population.

1.5 C4-*PEPC* and C4-*PPDK*

As already described in 1.4, C4-PEPC and C4-PPDK belong to the key enzymes of C4-photosynthesis (Sage, 2004), in which they play an important role as primary CO_2-fixing enzyme and for the regeneration of PEP by the phosphorylation of pyruvate (Langdale, 2011). The transcriptional activity of C4-*PEPC* and C4-*PPDK* is regulated by numerous internal and external signals. This adaptation allows the plant to react to different environmental conditions. In general, C4-genes, which are characterized by a higher expression than the corresponding C3-genes (Ku, Kano-Murakami and Matsuoka, 1996), are known to be induced by illumination. This can also be observed in the case of the genes C4-*PEPC* and C4-*PPDK*, which are both light-inducible and mesophyll-specific (Sheen, 1999). Additionally, nitrogen availability and sugar accumulation have also been demonstrated to influence C4-*PEPC* and C4-*PPDK* gene expression (Sheen, 1999; Hibberd and Covshoff, 2010; Horst, Heimann and Peterhansel, 2013).

It is supposed that the C4-isoforms of *PEPC* and other photosynthetic genes have evolved from C3-precursors that already possessed the necessary genetic predispositions for functional activity in the C4-mechanism (Sage, 2004; Wang *et al.*, 2009). The duplication of whole genomes, as well as individual genes is thought to have played an important role in the evolution of C4-photosynthesis. Subsequently, gene redundancy and alternative splicing may have contributed to functional innovations determining the role of the newly evolved isoforms in C4-photosynthesis (Monson, 2003; Wang *et al.*, 2009). An example can be found in the core family members of the *Chloridoideae*, in which C4-*PEPC* evolution was made possible by a gene duplication of a *PEPC* isoform. Neofunctionalization finally led to the development of C4-features in the duplicated gene (Christin *et al.*, 2007; Christin *et al.*, 2008). Therefore, it does not surprise, that for example in the case of C4-PEPC, plants do not solely possess the C4-isoform of the enzyme, but additional non-photosynthetic isoforms that fulfill different physiological functions (Latzko and Kelly, 1983; Kai *et al.*, 1999). In maize and sorghum for example, it was possible to identify one C4-isoform of *PEPC*, as well as accompanying further isoforms that do not play a role in C4-photosynthesis (Kawamura *et al.*, 1992). Like C4-*PEPC*, C4-*PPDK* is supposed to have developed from ancestral C3 precursor genes. Unequal recombination which led to repetitive sequences has probably contributed to this development. As a consequence, the ancestral C3 precursor genes gained a new intron, transit peptide and promoter, which allowed the C4-specific characteristics of the product (Glackin and Grula, 1990; Sheen, 1991, 1999). *PPDK* expression is based on a dual promoter system which has been discovered in both maize and rice. This system was also observed in the dicotyledonous species *Flaveria* (Matsuoka, 1995). Maize C4-*PPDK* shows great similarity with a cytosolic *PPDK*, which represents the ancestral gene

(Sheen, 1999). This can be explained by the fact that both can be found at the same gene locus, where they are transcribed from different initiation sites. Therefore, the two gene products are regulated by the activity of distinct promoters. This results in the expression of the C4-specific isoform of *PPDK* and the ancestral cytosolic isoform (Sheen, 1991, 1999; Matsuoka, 1995). The two isoforms are characterized by different sizes, as the C4-isoform contains an additional, first exon which encodes the chloroplast transit peptide and which cannot be found in the cytosolic isoform. Apart from this exon, the two isoforms are almost identical with regard to their coding sequence. In the C4-isoform, a 5 kb intron is located between the common sequence of the two isoforms and the transit peptide of C4-*PPDK*. It is characterized by repetitive sequences (Sheen, 1991, 1999). While the promoter of the cytosolic isoform shows ubiquitous activity, the C4-promoter is responsible for leaf-specific expression and the reaction towards illumination (Sheen, 1999). Apart from these two isoforms, a second cytosolic *PPDK* is known, which is very similar to the first one with regard to the coding region and the 5´region (Sheen, 1991).

One example for internal conditions controlling transcriptional activity has already been given, as the expression of a specific gene is dependent on the cell-type at question. C4-*PEPC* and C4-*PPDK* transcription is for example restricted to mesophyll cells (Sheen, 1999). While the mesophyll-specific expression of C4-*PEPC* is caused by the sequence region around -600 bp relative to the TIS, the responsible region for mesophyll-specific expression of C4-*PPDK* is located -300 bp relative to the TIS (Sheen, 1991; Taniguchi *et al.*, 2000). Apart from cell-type-specific expression, organ-specificity was also reported, like in the case of C4-*PPDK*, for which distinct *cis*-acting elements have been identified, which regulate leaf-, stem- or root-specific expression (Sheen, 1999). In addition to internal conditions, external stimuli do also play a vital role for the transcriptional activity of C4-genes. Illumination, nitrogen availability and sugar accumulation are important external factors that contribute to the determination of the degree of transcriptional activity in plants (Sheen, 1999; Hibberd and Covshoff, 2010; Horst, Heimann and Peterhansel, 2013), as mentioned above. The sequence region -300 bp relative to the TIS, which regulates the mesophyll-specific expression of C4-*PPDK*, also contributes to the genes regulation in response to illumination and for leaf specificity (Sheen, 1999). In this context, the light-inducible expression of C4-*PPDK* has been demonstrated to be uncoupled from the developmental stage of the leaf, but shown to be instead in part connected to the development of chloroplasts (Sheen, 1991). Two expression programs are responsible for this light-dependent expression pattern. They are caused by the presence of independent upstream regulatory elements. These elements do only play a role in leaves, but not in roots and stems, which explains the enzymes leaf-specificity (Sheen, 1999). In fact, Dong *et al.* (2016) reported a 290 or 130 times, respectively, higher expression rate for the C4-isoform than the cytosolic *CyPPDK1* isoform of *PPDK* (Dong *et al.*, 2016). Apart from light-inducible and organ-specific expression, a diurnal rhythm

has been reported for the activity of C4-*PPDK.* While the influence of a potential diurnal rhythm on the transcriptional activity of C4-*PPDK* has not yet been investigated, it is known that a bifunctional regulatory protein (RP) inactivates the enzyme in the dark. This process is caused by an ADP-dependent phosphorylation of the amino acid threonine 456 (in maize). In the light, the same amino acid is dephosphorylated again, which activates C4-*PPDK* (Hatch, 1987, 1997; Chastain *et al.*, 1997). Concerning C4-*PEPC* on the other hand, it has been shown that the mRNA abundance is characterized by a diurnal rhythm (Thomas *et al.*, 1990). This was also demonstrated by Horst *et al.* (2009). The mRNA and hnRNA level of C4-*PEPC* in maize vary throughout the day. This was also observed for acetylation levels. Notably, the declining transcriptional activity during the diurnal rhythm was independent of acetylation levels. If deacetylation was inhibited by TSA treatment, the transcriptional level was nevertheless reduced already during the illumination period (Horst *et al.*, 2009). When maize was exposed to constant illumination for three days, the variation in transcriptional activity and acetylation levels was maintained, but slowly declined. Notably, the decline in acetylation levels started earlier as the reduction of transcriptional activity, which still demonstrated high activity and variation at the second day of constant illumination, while acetylation levels already stayed constant. Therefore, it has been shown that diurnal gene regulation of C4-*PEPC* is controlled by a circadian rhythm independent of illumination and darkness. The diurnal variations in transcriptional activity are further independent of promoter acetylation (Horst *et al.*, 2009).

Concerning the regulation in response to illumination and accompanying light induction there are different theories explaining C4-*PEPC* promoter activation. It has been suggested that the light induction of C4-*PEPC* might be indirectly caused by the light induction of chloroplast biogenesis. Consequentially, an inhibition of chloroplast protein synthesis also leads to an inhibition of C4-*PEPC* promoter activity (Sheen, 1999; Kausch *et al.*, 2001). In an independent theory it has been suggested that a phytochrome-mediated response might be responsible for C4-*PEPC* light induction (Thomas *et al.*, 1990; Ma and Feng, 1993). Apart from illumination, the availability of nitrogen has also been reported to have a strong impact on the transcriptional activity of C4-*PEPC*. While the expression of C4-*PEPC* is impaired in plants that are suffering from nitrogen-depletion, supplying them with nitrate, ammonium or glutamine can reverse this effect and increase C4-*PEPC* expression again (Sugiharto and Sugiyama, 1992; Sugiharto *et al.*, 1992; Suzuki *et al.*, 1994; Sheen, 1999). In this context, cytokinin has been shown to play a major role in signal transduction and activates C4-genes in leaves, if nitrogen is available (Sakakibara *et al.*, 1998).

1.6 The regulation of transcriptional activity and gene expression

The example of cell-type specific expression of photosynthetic genes engaged in the C4-mechanism illustrates the fact that genes have to be differentially regulated, for example to meet the requirements of a specific cell-type. Apart from this, the necessity to up-regulate, down-regulate or even repress a certain gene is further determined by internal or external conditions, as described in 1.1. Different situations are characterized by specific demands that force an organism to adapt. The situation can for example be determined by internal conditions, like the organ or tissue in which a cell is located, or by external influences, like the availability of nutrients and light, as described above. Consequentially, each cell expresses a different set of enzymes, dependent on the specific situation it faces. Therefore, not all of the genes contained in a cell are transcribed at the same time or in the same amount. As a consequence, complex modes of gene regulation are required to enable an organism to adapt to a specific situation. One possibility to regulate the transcriptional activity of a gene and thereby adjust the amount of its product is the regulation on the chromatin level (Horst, Heimann and Peterhansel, 2013).

1.7 Chromatin

In order to organize deoxyribonucleic acid (DNA) in a way that enables the large molecule to be stored in the nucleus of a cell, it is organized in the form of chromatin. Chromatin consists of DNA associated with histone proteins. These basic proteins contain a high amount of the amino acid residues lysine and arginine which possess a positive charge (Nelissen *et al.*, 2007). The histone proteins form octamers of each two of the histone subunits H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999) or variants of these subunits (Ausió, 2006). 147 bp of DNA are wrapped around each of the histone octamers, comprising 14 contact points, which leads to high stability of the complex (Luger *et al.*, 1997; Kouzarides, 2007). Additionally, the histone subunit H1 interacts with the protein-DNA complex resulting in altogether 165 bp of DNA that are wound around the particle (Lewin, 2002).Together, DNA and histones form an array of disk-shaped structures (Kouzarides, 2007), which are described as nucleosomes (Kornberg, 1974). The nucleosomes are organized repetitively and located every 160 to 200 base pairs throughout the genome, thus forming a “beads-on-a-string” configuration (Hewish and Burgoyne, 1973; Olins and Olins, 1974; Venters and Pugh, 2009), whereby each of the beads represents a nucleosome and the N-terminal tails form a connection to the adjacent nucleosomes (Bannister and Kouzarides, 2011). Nucleosomes play a major role in packaging

of DNA-molecules in the nucleus, as under physiological conditions, they form a highly stable protein-DNA complex (Li, Carey and Workman, 2007). This interaction provides the basis of DNA condensation and enables further higher order organization of the DNA molecule, finally resulting in the characteristic form of chromosomes.

Apart from its importance in DNA packaging and storage, chromatin plays a major role in metabolic processes that are associated with DNA. First of all, the cellular transcription machinery has to overcome the structural barrier provided by chromatin (Venters and Pugh, 2009) for transcription initiation. This was already discovered in *in vitro* experiments by Lorch, LaPointe and Kornberg (1987), which demonstrated that transcription initiation was inhibited in the presence of nucleosomes (Lorch, LaPointe and Kornberg, 1987). In accordance to these early observations, experiments in *Saccharomyces* revealed that a depletion of histones on the contrary led to an increase of transcription (Han and Grunstein, 1988; Wyrick *et al.*, 1999). But apart from influencing the accessibility of genes to the transcriptional machinery, histones also provide a suitable basis for the interaction with activating and repressing effectors that influence the activity of a gene, like transcription factors. Chemical modifications of the amino acid residues of histone proteins are a major factor in these interactions, as these modifications can be recognized by other proteins (Seet *et al.*, 2006), thus providing a suitable basis for gene regulation, as will be explained in more detail.

1.8 Chromatin modifications

Covalent modifications of histone residues are a well-known means for the regulation of gene expression. By the use of specific antibodies or mass spectrometry, more than 60 amino acid residues on histone proteins have been characterized as being modified (Kouzarides, 2007). Acetylation and methylation of histone amino acid residues are two of the best studied covalent modifications of histones. Already in 1964, the acetylation and methylation of histones has been described in the context of gene regulation. By then it was supposed that modifications might have an impact on the inhibition of transcription caused by histones *in vivo*, as experiments revealed that acetylation of histones led to increased RNA synthesis (Allfrey, Faulkner and Mirsky, 1964) Indeed, acetylation is associated with actively transcribed genes and can be observed at a series of different lysine residues. Examples for histone acetylations which, among others, have already been investigated in detail are H3K9, H3K14, H3K18, H4K5, H4K8 and H4K12 (Kouzarides, 2007). Histone acetylations were found to be enriched at enhancer elements and promoters. At these locations, their presence might point to a role in facilitating the access of transcription factors to the DNA

(Wang *et al.*, 2008). Acetylations have for example been known to be associated with the cell cycle and cell division in roots of *Zea mays* and *Tulipa kaufmanniana* and in *Lilium* microsporocytes, already in the seventies of the 20th century (Nadler, 1976; Kononowicz, 1978; Nelissen *et al.*, 2007). H4 acetylation for example plays an important role in DNA-replication, thus contributing to cell cycle regulation. In this context, nucleolus organizers have been reported to demonstrate H4 acetylation patterns which are dependent on the phase of the cell cycle. While during mitosis, acetylation levels were high, the levels decreased during the S-phase (Jasencakova *et al.*, 2000). In another context, the activating effect of histone acetylations also plays an important role in cell fate acquisition (Nelissen *et al.*, 2007). An example can be found in the development of root hair cells. When histone deacetylases, which remove acetylations, are inhibited by a treatment with trichostatin A, gene positions that are usually associated with the suppression of root hair growth become hyperacetylated. This leads to the induction of root hair growth, underlining the activating role of histone acetylations in gene expression (Xu *et al.*, 2005).

Histone methylations can be predominantly found at lysine or arginine residues. In contrast to the acetylation of lysine residues, which alter the amino acid residues charge and reduce electrostatic interaction with the DNA (Dion *et al.*, 2005), the methylation of lysines does not lead to an altered charge of the histone protein. Another difference to acetylations is that the methylation state of a histone is not limited to just one methylation. Depending on the enzyme responsible for the methylation, lysines can show a mono-, di-, or tri-methylated state (Turner, 2005; Ng, Chandrasekharan and Hall, 2006; Nelissen *et al.*, 2007; Bannister and Kouzarides, 2011). A similar phenomenon can be observed in the case of arginine methylation, which can occur as mono-, symmetrical or asymmetrical dimethylation (Turner, 2005; Ng, Chandrasekharan and Hall, 2006). As they are not part of the current thesis, arginine methylations are not further described at this point. Histone methylations can for example be observed in the context of transcriptional repression. The initiation of silent heterochromatin and its maintenance are further regulatory mechanisms, in which histone methylation plays an important role (Nelissen *et al.*, 2007). Nevertheless, methylation can also be connected to activating processes, like in the case of the histone trimethylation H3K4me3 (Kouzarides, 2002). The connection between H3K4me3 and activating regulatory mechanisms has for example been observed in studies focusing on the mating type loci in fission yeast. In this context, silent heterochromatin, which makes up large stretches of about 20 kb, has been found to be rich in lysine residue 9 methylation. Lysine 4 methylation on the other hand could not be observed in these parts of the gene. The situation on transcriptionally active parts of the gene on the other hand, demonstrated a contrasting methylation pattern, as in these parts, lysine 4 was methylated instead of lysine 9 (Kouzarides, 2002). In 2013, Cui *et al.* demonstrated that the silencing of transposable elements (TE), which is performed to prevent mutagenesis caused by active transposition, is

impaired under increased histone trimethylation. The JMJ703 rice methyltransferase plays a crucial role in these silencing processes, as it removes H3K4 methylation marks. If the activity of the demethylase JMJ703 is impaired, the detectable levels of H3K4me3 consequentially increase. In parallel to the increased levels of the activating trimethyl mark, two retrotransposons have been observed to be reactivated (Cui *et al.*, 2013). H3K27me3 is known to play an antagonistic role towards the active chromatin mark H3K4me3 (Liu *et al.*, 2015). H3K27me3 is a repressive histone modification that is for example associated with dioxygenase genes (Charron *et al.*, 2009). It has recently been reported that in rice, the methyltransferase SDG701 specifically methylates H3K4 and is involved in developmental processes (Liu *et al.*, 2017). When the enzyme is over-expressed, rice flowering is promoted. A knockdown on the other hand, leads to delayed flowering processes. In this context, the increased H3K4 trimethylation has been observed to be associated with the increased expression of *Hd3a* and *RFT1*, which are rice florigens, thus underlining the activating role of H3K4me3 (Liu *et al.*, 2017).

Apart from acetylations and methylations of histone amino acid residues, a series of further histone modifications has been described. These are for example deiminations, in which an arginine residue is converted to citrulline, the modification by β-N-acetylglucosamine sugar residues, the mono- and poly-ADP ribosylation of arginine and glutamate residues, ubiquitylation, sumoylation, phosphorylation, as well as histone tail clipping and histone proline isomerization (Nelissen *et al.*, 2007; Bannister and Kouzarides, 2011). Altogether the numerous modifications that are known to alter histone amino acid residues do not only take part in the regulation of transcriptional activity, but are also known to be involved in processes like DNA repair, replication or recombination (Nelissen *et al.*, 2007; Bannister and Kouzarides, 2011).

The high number of different modifications that can be introduced at histone residues already indicates the complexity of regulatory events taking place on the chromatin level. Furthermore, it gives an idea of the various possibilities to fine-tune responses to external or internal stimuli. The degree of complexity is further increased, as modifications are known to influence each other (Jenuwein and Allis, 2001; Fischle, Wang and Allis, 2003; Berger, 2007). Thus, a high degree of regulatory fine-tuning can be achieved with regard to transcriptional activity. This enables an organism to adapt to various different situations. In this context, the modification events that are associated with activation are acetylations, methylations, phosphorylations and ubiquitylation. In the case of transcriptional repression, methylations, ubiquitylations, sumoylations, deiminations and proline isomerizations have been observed (Kouzarides, 2007; Bannister and Kouzarides, 2011). Nevertheless, the situation in which a specific modification occurs, as well as the location where it is found, influences the activating or repressing effect of said modification. Therefore, it should be

considered that a given modification can show both, activating or repressing potential, depending on the situation the organism is experiencing and the modifications location on the gene (Kouzarides, 2007).

The crosstalk and interaction between different modifications can for example take place in a competitive way. This is the case if a specific modification side is targeted by several modifying enzymes and thus subjected to different modifications (Bannister and Kouzarides, 2011). Apart from this competitive antagonism, modifications can also be dependent from each other or demonstrate cooperation with regard to the recruitment of regulatory factors (Jenuwein and Allis, 2001; Fischle *et al.*, 2005; Berger, 2007; Bannister and Kouzarides, 2011). A given histone modification can further be able to influence the binding of a protein to another histone modification on the same gene. For example the binding can be impaired by the presence of a specific adjacent modification. HP1 is known to bind to mono- or dimethylated forms of H3K9. However, during mitosis, the phosphorylation of serine 10 on histone H3 disrupts this binding. Finally, modifications also influence the activity of their protein binding partners (Fischle *et al.*, 2005; Bannister and Kouzarides, 2011).

A well studied example for the complex interplay between different types of histone modifications and their impact on gene expression can for example be seen in the control of flowering time (Nelissen *et al.*, 2007). Various external signals, like the illumination period and temperature, have to be integrated to determine the correct time for the initiation of the flowering process. An antagonist to flowering promoting external signals is the autonomous pathway which is responsible for a constitutive repression of the process (Nelissen *et al.*, 2007). The integration of these signals helps the plant to induce the flowering process at a time point which provides optimal conditions for its reproductive success (He and Amasino, 2005). In *Arabidopsis*, this repression is achieved by the contribution of the *Flower Locus C* (*FLC*), which plays a major part in the flowering process, as well with regard to activating external signals, as to the repressive autonomous pathway. Both, H3K4me3 and histone acetylation activate *FLC* expression. A high expression level of this transcription factor leads to a delay in flowering. Histone deacetylation and H3K9/H3K27 dimethylation on the contrary are associated with low *FLC* expression levels (He and Amasino, 2005). The delay in flowering transition which is caused by high expression levels of *FLC* is for example achieved by a repressive effect of *FLC* on *SOC1* and *FT*, which play a role as flowering-time integrators (Hepworth *et al.*, 2002). H3K4me3 and histone acetylations contribute to the regulation of the *FLC* expression level (He, Michaels and Amasino, 2003; Bastow *et al.*, 2004; Sung and Amasino, 2004). In yeast, the PAF1 complex is responsible for the recruitment of the H3K4 methyltransferase SET1 which establishes a trimethylation of H3K4 (Krogan *et al.*, 2003; Ng *et al.*, 2003; He and Amasino, 2005). An equivalent to the yeast Paf1 complex has been characterized in *Arabidopsis*, comprising EARLY FLOWERING 7

(ELF7), EARLY FLOWERING 8 (ELF8) and VERNALIZATION INDEPENDENCE 4 (VIP4). In elf7 and elf8 mutants, a reduction of H3K4me3 on the *FLC* locus has been reported (He, Doyle and Amasino, 2004). This reduction of H3K4me3 is accompanied by reduced *FLC* expression levels and consequentially early flowering (He and Amasino, 2005) which underlines the association of H3K4 trimethylation with increased *FLC* expression and concomitant delay of flowering. As mentioned above, apart from H3K4me3, histone acetylations are also involved in high expression levels of *FLC*, while deacetylation leads to a reduction in *FLC* expression (Nelissen *et al.*, 2007). For example, FLOWERING LOCUS D and FVE have been demonstrated to show similarities to the histone deacetylase complex known in mammals and are able to deacetylate *FLC* chromatin which leads to reduced expression rates (He and Amasino, 2005).

Today, two different models have been described, which explain the influence of histone acetylation on transcriptional activity. The first way in which acetylation regulates the activity of a certain gene is by influencing the structural properties of chromatin. As the lysine residues of histone proteins have a positive charge, they contribute to the interaction of the protein with the negatively charged DNA-molecule. Being subjected to acetylation, the lysine residue loses this positive charge. Due to the charge neutralization, the interaction between the histone protein and the DNA is weakened. Consequentially, the nucleosomes structure is less dense. This enables an easier access of the transcription machinery to the DNA-sequence at question. This effect is reversed, when the acetylation is removed by histone deacetylases. The neutralization of positively charged lysine side chains can no longer be observed and the strength of the proteins interaction with the DNA-molecule increases (Bannister and Kouzarides, 2011). Apart from this, structural properties of chromatin are regulated by the recruitment of remodeling complexes by histone modifications. In this context, the hydrolysis of ATP provides energy for the relocation of nucleosomes (Bannister and Kouzarides, 2011). Secondly, histone acetylations represent binding surfaces for activating or repressing factors that influence the transcriptional activity. Thereby a complex regulatory network is established, as single acetylation events, as well as groups of histone acetylations demonstrate co-regulation or anti-correlation (Kurdistani, Tavazoie and Grunstein, 2004).

These two independent explanatory approaches for the regulation of gene expression on the chromatin level led to the development of two distinct models describing the integration of external and internal stimuli into a suitable response. In this context, the aforementioned neutralizing effect of histone acetylations on the electrostatic interaction between positively charged lysine residues and the negatively charged DNA molecule (Dion *et al.*, 2005) is the basis for the charge neutralization model. The charge neutralization model defines the integration of external information as being based on the acetylation of histone residues and, therefore, on the weakened interaction between

histones and DNA. This means that further modifications would only play a role in regulating the response to this information (Horst, Heimann and Peterhansel, 2013). As it was also possible to observe a more complex regulatory network, in which individual histone modifications are recognized by transcription factors and additionally influence each other (Jenuwein and Allis, 2001; Berger, 2007) a histone code model has been suggested as an alternative to the charge neutralization model. The histone code model underlines that specific signals determine the presence or absence of histone modifications, which are, therefore, a suitable means for the integration and storage of information (Horst, Heimann and Peterhansel, 2013).

1.9 Influences on transcriptional activity and histone modifications regulating the expression of C4-*PEPC*

To adapt the expression of a specific gene product to external and internal necessities, a complex fine-tuning of regulatory events is indispensable, as described above. In the case of C4-genes, like C4-*PEPC* these events have already been well-studied and a series of internal and external stimuli are known to take part in the regulation.

As already described in 1.8, increased acetylation levels are in general associated with actively transcribed genes. Illumination is one of the various external stimuli which regulate C4-*PEPC* expression. When a plant is illuminated, the influence of light strongly induces the transcriptional activity of C4-*PEPC* (Sheen, 1999; Offermann *et al.*, 2006, 2008; Danker *et al.*, 2008). While the nucleosome density does not show a strong reaction to light (Offermann *et al.*, 2006), exposure to light leads to an increase of H3 and H4 acetylation at the promoter and at the 5´ part of the transcribed region (Choi and Howe, 2009) which accompanies the increased expression rate of the photosynthetic gene. Concerning individual lysine residues, the increase of acetylation under the influence of light has been demonstrated for example for the histone modifications H3K9ac and H4K5ac. Both show a 3 to 10-fold induction (depending on the position), in maize plants exposed to illumination. For both modifications, maximal acetylation levels are found on the promoter. In the case of H3K9ac, a peak was observed at position -2000 bp relative to the transcription initiation site (TIS) which indicates that the distal promoter might play an important role in the light dependent regulation of the gene. While the level of H4K5 acetylation also increases upon illumination, its distribution is more even than in the case of H3K9ac (Offermann *et al.*, 2008). Additionally, both modifications are known to show a diurnal rhythm (Horst *et al*. 2009), which corresponds to the observation that the mRNA and hnRNA level of C4-*PEPC*, as well as the RNA Polymerase II abundance

in the coding region of C4-*PEPC* is low at night and high during the day (Thomas *et al.*, 1990; Horst *et al*. 2009). The acetylation of H3K18 does not show strong reactions to illumination, in contrast to H3K9ac and H4K5ac. H3K18ac is distributed evenly over the gene, but shows reduced levels behind the TIS (Offermann *et al.*, 2008).

The participation of the two distinct cell-types in the C4-mechanism requires a cell-type specific expression of C4-genes at their site of activity. C4-*PEPC* has not only been demonstrated to be light-inducible, but is also known to be expressed in mesophyll cells exclusively (Sheen, 1999; Kausch *et al.*, 2001). Consequentially, histone modifications responsible for the cell-type specific expression of C4-genes also show a cell-type specific distribution. In this context, methylation states of histones are of special interest. They are dependent on cell-type specific developmental signals which lead to a characteristic distribution of methylation patterns. It has been demonstrated that H3K4me3 is primarily found in mesophyll cells, while H3K4me2 is specific for bundle sheath cells. Therefore, the switch from mono- and di- to trimethylation of H3K4me3 was identified as important factor for potentiating C4-*PEPC* for cell-type specific expression. Additionally, H3K4me3 demonstrates an organ specific distribution pattern, as it is only detectable at very low levels in roots, while leaves show high H3K4me3 levels (Danker *et al.*, 2008). This means that the trimethylation H3K4me3 in C4-plants is a modification mark that is specifically involved in the determination of the organ or cell-type in which a certain gene can be transcribed. In this context, it potentiates the gene for activation. Due to the presence of H3K4me3, the possibility of transcriptional activity is in principle given, even if the gene is actually not actively transcribed. Additional modifications, like increased H3K9ac levels in illuminated leaves, will subsequently determine whether transcription is induced or not (Horst, Heimann and Peterhansel, 2013). Summarizing, the mesophyll-specific and light-inducible expression of C4-*PEPC* can be simplified explained based on the characteristic behavior of H3K4me3 and H3K9ac. First of all, H3K4me3 presence determines whether the gene can be activated at all in a specific organ or cell-type (Danker *et al.*, 2008). Presumed that the gene is in fact poised by H3K4me3 for transcriptional activation in a given cell-type, activation will take place under the influence of light, when an additional increase in H3K9ac takes place (Offermann *et al.*, 2008; Horst, Heimann and Peterhansel, 2013). In darkened mesophyll-cells on the contrary, the gene would still be poised for activation, due to the presence of H3K4me3, but nevertheless transcriptional activity would be low, as well as H3K9ac levels. This pattern was not only described for C4-*PEPC*, but also in other C4-genes, like *Glk*1, *Cp*24, *Cp*26, *Cp*29 (Horst, Heimann and Peterhansel, 2013), C4-*CA*, C4-*PPDK*, C4-*ME*, C4-*PEPCK* and C4-*RbcS2*, which demonstrated increased levels of H3K9ac in leaves exposed to illumination while levels stayed low in darkened leaves. Additionally, H3K4me3 showed high levels in mesophyll cells, but not in bundle sheath cells, independent of the illumination status (Heimann *et al.*, 2013). This phenomenon was also observed in sorghum and *Setaria italica* (Heimann *et al.*, 2013;

Horst, Heimann and Peterhansel, 2013). Accordingly, the trimethylation of H3K4 is independent of transcription initiation. H3K4me3 is not removed or reduced upon prolonged darkness or upon pharmaceutically achieved inhibition of transcription.

Apart from the illumination status and the cell-type, the availability of nutrients like nitrogen also plays an important role in the expression of photosynthetic genes. It has been shown that the mRNA and protein levels of photosynthetic enzymes like PEPC, PPDK, and RUBISCO are regulated in response to the availability of nitrogen. For example, maize plants suffering from nitrogen depletion have been demonstrated to show decreased *PEPC* levels (Sugiharto *et al.*, 1990). If nitrogen is added to the plant exogenously, the effect of nitrogen starvation is reversed. In this case, the levels of mRNA and protein increase. In this context, the increase of *PEPC* mRNA due to an addition of nitrogen, ammonium and glutamate has been demonstrated (Sugiharto and Sugiyama, 1992). Sugiharto *et al.* (1992) examined the mechanisms that are responsible for the regulation of *PEPC* expression by nitrogen availability. When maize leaves were detached from the plant, a decrease in *PEPC* mRNA levels was observed. This decrease could be inhibited by administering zeatin, a naturally occurring cytokinin, to the detached leaves. The effect could also be observed, when the synthetic cytokinin benzyladenine was applied instead of zeatin. Thus is has been concluded that the regulation of nitrogen dependent expression of *PEPC* is achieved by cytokinin (Sugiharto *et al.*, 1992). Cytokinins are synthesized in the root (Feldman, 1979). They are then transported to the distant photosynthetic cells of the plant, where they up-regulate the expression of C4-*PEPC* and other photosynthetic genes. The importance of zeatin for the involved signaling events was underlined by experiments examining the *in vitro* transcription rate of *PEPC* under the influence of zeatin and under the influence of nitrogen sources. Zeatin alone was already able to increase the *in vitro* transcription rate, while this effect could not be observed when nitrogen sources where applied alone. When zeatin and nitrate were applied in combination, the transcription rate was higher than in the case of zeatin addition alone. Therefore, zeatin is able to up-regulate the expression of C4-*PEPC*, dependent on the level of nitrogen availability. This nitrogen-dependent increase in *PEPC* expression was independent of illumination (Suzuki *et al.*, 1994). The influence of nitrogen availability or depletion on the distribution and level of histone modifications has been studied in detail. Offermann *et al*. (2008) examined changes in the acetylation of several histone modifications, including H3K9ac, H3K18ac and H4K5ac, in plants suffering from nitrogen depletion. Nitrogen availability influences the acetylation levels of said modifications, among others. According to previous knowledge about the reaction of *PEPC* transcription towards nitrogen availability, nitrogen depletion also led to a strong reduction in hnRNA levels. It can, therefore, be concluded that the transcriptional level and acetylation at the distal promoter are coregulated for C4-*PEPC* with regard to nitrogen availability (Offermann *et al.*, 2008).

In this context, it has to be noted that the proximal and distal C4-*PEPC* promoters are differentially regulated with regard to nitrogen availability. At the proximal promoter and at the beginning of the transcribed region, nitrogen depletion did not provoke changes in acetylation levels. Modifications on the distal promoter on the contrary showed a 3 to 5 fold reduction of acetylation levels. This example shows that regulatory events at the distal and the proximal promoter follow different principles with regard to their reaction to external stimuli. Here, both the charge neutralization model and the histone code model can be applied, depending on the position. At the distal promoter, the observed histone modifications demonstrated reaction towards different stimuli, like for example nitrogen depletion or repression by metabolites (Offermann *et al.*, 2008; Horst, Heimann and Peterhansel, 2013). Therefore, for the explanation of regulatory events at the distal C4-*PEPC* promoter, the charge neutralization model (Dion *et al.*, 2005), in which histone modifications represent means to regulate the promoters response function, has to be considered (Horst, Heimann and Peterhansel, 2013). Modifications at the core promoter on the contrary reacted only to specific stimuli. As explained above, H3K9ac (similar to H4K5ac), is regulated by the influence of light, while H3K4me3 is not light-inducible, but instead shows a cell-type specific distribution pattern (Danker *et al.*, 2008; Offermann *et al.*, 2008; Horst, Heimann and Peterhansel, 2013). Therefore, it can be concluded that modifications at the core promoter do not follow the charge neutralization model, but can instead be explained by applying the histone code model in which distinct histone modifications are integrated to regulate a specific response to a given situation (Berger, 2007; Horst, Heimann and Peterhansel, 2013).

1.10 Influences on transcriptional activity and histone modifications regulating the expression of C4-*PPDK*

An important stimulus for the regulation of C4-*PPDK* is illumination. In previous studies, it has been observed that the nucleosome density on C4-*PPDK* increased under prolonged darkness in re-etiolated plants. The reaction of histone acetylations to illumination and prolonged darkness was evaluated for the proximal promoter (at position -150 bp relative to the TIS) and for the distal promoter (at position – 760 bp relative to the TIS) (Horst, 2009). In this context, the acetylation level of the individual lysine residues H3K9, H3K18 and H4K5, which are also part of the present thesis, has been measured. Concerning the acetylation level of H3K9, both promoter regions of C4-*PPDK* demonstrated differences between illuminated and re-etiolated plants. At both promoter regions, the absence of light led to a decrease in H3K9ac levels. This effect was similar to what was already known for C4-*PEPC*. In contrast to this, the acetylations level at H3K18 increased under darkness at both, the proximal and the distal promoter. In the case of H4K5ac, different effects were observed at the distal and the proximal promoter. While the acetylations level decreased in re-etiolated plants at the distal promoter, it stayed constant at the proximal promoter (Horst, 2009). Dong *et al.* (2016) further reported that both the C4-isoform of *PPDK* and the cytosolic isoform *CyPPDK*1 which is, with regard to the coding region, nearly identical to C4-*PPDK*, showed a 290 or 130 times higher expression rate in leaves, than in other organs, like roots and stems. In developing seeds, it was not possible to detect either of the two *PPDK* isoforms at all. The second cytosolic *PPDK* isoform, *CyPPDK2* on the other hand showed a highly increased mRNA abundance in developing seeds, in comparison to leaves and roots (Dong *et al.*, 2016). This organ-dependent expression pattern was mirrored in the distribution of H4K5 and H3K9 acetylation. Highest acetylation levels for H3K9 were measured in the leaves, while the levels were lower in stems, roots and seeds. For H4K5ac, the acetylation level was highest in roots and leaves. Notably, this difference was only observed in C4-*PPDK*, while the non-C4-isoforms *CyPPDK1* and *CyPPDK2* showed no differences with regard to the organ (Dong *et al.*, 2016).

1.11 Aim of the thesis

As already described in 1.1, approaches to introduce the C4-mechanism into C3-crops, with special regard to rice would be highly valuable, as they might offer a possible solution for already existing nutritional problems and their possible increase due to the climate change. With regard to possible solutions to introduce the C4-mechanism into rice, the question whether C4-genes would be regulated in the desired way, arises. If a functional C4-mechanism shall be achieved in C3-crops like rice, it is of great importance that the required genes are regulated in a similar way as in regular C4-plants. In this context, the present thesis concentrates on the photosynthetic C4-*PEPC* gene. With regard to light induction, C4-*PPDK* was also included in the experiments. As described above, the regulation of C4-*PEPC* and C4-*PPDK* in maize with regard to the occurrence of specific histone modifications has already been examined in detail. For the comparison of the appearance of a series of chromatin modifications in maize and rice, transgenic rice, expressing C4-*PEPC* or C4-*PPDK* was used in experiments of the present thesis. For a functional C4-mechanism in C3-plants, the reaction of photosynthetic C4-genes in transgenic rice towards different stimuli and to the complex interplay of these stimuli might have to correspond to the situation in an actual C4-plant. If this holds true, the similarity of the reaction in transgenic rice to the situation in an actual C4-plant would be a necessary prerequisite for a fully functional C4-photosynthesis in former C3-crops. In this case, it is important to know whether the genes of C4-photosynthesis are functional in C3-plants at all and if they are regulated in a similar way. The aim of the present thesis was to evaluate the regulatory features of C4-genes that are expressed in C3-plants. Therefore, the histone modifications H3K9ac, H3K18ac, H4K5ac and H3K4me3 have been analyzed in this thesis in maize and transgenic rice, with regard to their distribution and abundance under the influence of different stimuli. The aim of this approach was to evaluate if the histone modifications that are present in a given situation in the donor plant maize show a corresponding distribution and abundance in transgenic rice in a corresponding situation. For this purpose the reaction to illumination, the occurrence of a diurnal regulation, as well as the reaction to nitrogen depletion have been investigated. If the modifications are in fact important for the regulation of C4-specific gene expression, similarity between the two sepcies would indicate that a functional C4-mechanism would in general be possible in the C3-crop.

2 Material and Methods

2.1 Material

2.1.1 Chemicals and consumables for the general use

The used chemicals were in general characterized by a purity degree of at least p.a. Chemicals and consumables were purchased from the following suppliers:

Table 2.1: Chemicals and consumables used throughout the experiments.

Chemical or consumable	**Supplier**
Agarose Basic	AppliChem Panreac ITW, Darmstadt
Betaine	Sigma, Taufkirchen
cOmplete™ Protease Inhibitor Cocktail	Roche, Basel
dNTPs	Thermo Scientific, Waltham (USA)
EDTA	AppliChem, Darmstadt
Formaldehyde	AppliChem, Darmstadt
KNO_3	AppliChem, Darmstadt
Glycine	AppliChem Panreac ITW, Darmstadt
Lithium chloride (LiCl)	AppliChem Panreac ITW, Darmstadt
Magnesium chloride ($MgCl_2$)	AppliChem, Darmstadt
Magnesium chloride, 50 nM for qPCR	Invitrogen, Carlsbad (USA)
β-Mercaptoethanol	AppliChem, Darmstadt
Nonidet® P40	AppliChem, Darmstadt
Phenylmethylsulfonylfluoride (PMSF)	Carl Roth GmbH and Co. KG, Karlsruhe
Protein A agarose	Roche, Basel
SDS 20 %	AppliChem Panreac ITW, Darmstadt
Sodium butyrate	Aldrich Chemistry, St. Louis (USA)
Sodium chloride (NaCl)	AppliChem, Darmstadt
Sodium deoxycholate	Merck, Darmstadt

Sucrose	Carl Roth GmbH and Co. KG, Karlsruhe
Tris ultrapure	AppliChem Panreac ITW, Darmstadt
Triton X-100	Merck, Darmstadt
Zeatin	Sigma

2.1.2 Instruments and supplies

Table 2.2: Instruments and supplies used throughout the experiments.

Instruments or supplies	**Supplier**
BB-XL2 GroBank	CLF Plant Climatics, Wertingen
BB-XL3 GroBank	CLF Plant Climatics, Wertingen
Bioruptor™ Twin	Diagenode, Denville (USA)
BioTek Gen5™ Take3™ Module	BioTek, Winooski (USA)
BioTek Take3™ Multi-Volume Plate	BioTek, Winooski (USA)
Centrifuge 5810R	Eppendorf, Hamburg
Centrifuge 5415R	Eppendorf, Hamburg
DIAMOND Sterilized Filter Tips DF30 Diamond Tipack, 2.0 µl-30 µl	Gilson, Inc., Middleton (USA)
DIAMOND Sterilized Filter Tips DFL10 Diamond Tipack, 0.1 µl-10 µl	Gilson, Inc., Middleton (USA)
DIAMOND Sterilized Filter Tips DF200 Diamond Tipack, 20 µl-200 µl	Gilson, Inc., Middleton (USA)
DIAMOND Sterilized Filter Tips DF1000 Diamond Tipack, 100 µl-1000 µl	Gilson, Inc., Middleton (USA)
DURAN® Erlenmeyer flask, 250 ml	DURAN Group GmbH, Wertheim/Main
Einheitserde Classic Profi Substrat, Topferde CLT	Einheitserdewerk Uetersen Werner Tantau GmbH and Co. KG, Uetersen
Einheitserde Classic Profi Substrat, Vermehrungssubstrat VM	Einheitserdewerk Uetersen Werner Tantau GmbH and Co. KG, Uetersen
Electrophoresis chamber	Biozym, Hessisch Oldenburg
Eppendorf Tubes®, 5.0 ml	Eppendorf, Hamburg
Fumehood	Köttermann GmbH and Co. KG, Uetze
Greiner Bio-One CELLSTAR® Centrifuge Tubes,	Greiner Bio-One International GmbH,

50 ml	Kremsmünster, Oberösterreich
Heidolph Reax2 overhead shaker	Heidolph Instruments GmbH and Co. KG, Schwabach
IKA® RH basic 2 magnetic stirrer	IKA®, Staufen im Breisgau
IKA® VXR basic Vibrax® orbital shaker (Type VX2E)	IKA®, Staufen im Breisgau
Incubator	Memmert, Schwabach
INTAS Gel-Imager	INTAS, Göttingen
KNF LABOPORT® vacuum pump	KNF Neuberger, Village Neuf (France)
MicroAmp™ Optical Adhesive Film	Thermo Scientific, Waltham (USA)
Micro Tubes, 1.5 ml	Sarstedt, Nümbrecht
Miracloth	Merck Millipore, Billerica (USA) VWR, Radnor (USA)
Mortar (55-00, 55-2, 55,3)	W. Haldenwanger GmbH and Co., Berlin
Peleus ball	Carl Roth GmbH and Co. KG, Karlsruhe
Pestle (56-00, 56-1, 56-3)	W. Haldenwanger GmbH and Co., Berlin
PIPETMAN Neo® P1000N	Gilson, Inc., Middleton (USA)
PIPETMAN Neo® P200N	Gilson, Inc., Middleton (USA)
PIPETMAN Neo® P20N	Gilson, Inc., Middleton (USA)
PIPETMAN Neo® P10N	Gilson, Inc., Middleton (USA)
Precision scale 572	KERN and SOHN GmbH, Balingen-Frommern
qPCR 96-well high sided, low profile Plates	Thermo Scientific, Waltham (USA)
Safe-Lock Tubes, 2.0 ml	Eppendorf, Hamburg
Serological Rotilabo®-pipettes, blue 5.0 ml	Carl Roth GmbH and Co. KG, Karlsruhe
Serological Rotilabo®-pipettes, orange 10.0 ml	Carl Roth GmbH and Co. KG, Karlsruhe
Serological Roltilabo®-pipettes, red 25.0 ml	Carl Roth GmbH and Co. KG, Karlsruhe
StepOnePlus™ Real-Time PCR System	Applied Biosystems, Foster City (USA)
Synergy™ Mx Microplate Reader	BioTek, Vermont (USA)
Take3™ Multi-Volume Plate	BioTek, Vermont (USA)
Vacuum bell jar	DURAN Group GmbH, Wertheim/Main
Vacuum controller V850	BÜCHI Labortechnik GmbH, Essen
Vacuum pump V700	BÜCHI Labortechnik GmbH, Essen
Water bath GFL	GFL Gesellschaft für Labortechnik GmbH, Burgwedel
Water bath GFL1038	GFL Gesellschaft für Labortechnik GmbH,

Burgwedel

2.1.3 Software and internet applications

Table 2.3: Software and internet applications used for research and analysis.

Software and internet applications	**Supplier**	**Purpose**
Clone Manager 9	Scientific and Educational Software, Denver (USA)	Analysis of nucleic acid sequences and primer design
Gen5™ Microplate Reader and Imager software, version 1.11.	BioTek, Vermont (USA)	Measurement of DNA and RNA concentrations, quality control
Gramene	Cold Spring Harbor Laboratory, Oregon State University, EMBL-EBI (http://www.gramene.org)	Database
The European Bioinformatics Institute (EMBL-EBI)	EMBL-EBI (http:www.ebi.ac.uk)	Database
National Center for Biotechnology Information homepage (NCBI)	National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)	Database
Oligo Calculator	http://www.basic.northwestern.edu /biotools/OligoCalc.html	Oligonucleotide properties calculator
Phytozome	Joint Genome Institute (JGI) (https://phytozome.jgi.doe.gov/ pz/portal.html)	Database
StepOne™ software, version 2.3	Applied Biosystems, Foster City (USA)	Software for the StepOnePlus™ Real-Time PCR System

2.1.4 Antibodies used for chromatin immunoprecipitation (ChIP)

Table 2.4: Antibodies used for chromatin immunoprecipitation.

Antibody	Supplier	Purpose
Anti-acetyl-Histone H3 (Lys9), polyclonal antibody (catalogue number 07-352)	EMD Millipore Corporation, Temecula (USA)	For ChIP, directed against acetylated lysine 9 of histone 3
Anti-acetyl-Histone H3 (Lys18), polyclonal antibody (catalogue number 07-354)	EMD Millipore Corporation, Temecula (USA)	For ChIP, directed against acetylated lysine 18 of histone 3
Anti-acetyl-Histone H4 (Lys5), polyclonal antibody (catalogue number 07-327)	EMD Millipore Corporation, Temecula (USA)	For ChIP, directed against acetylated lysine 5 of histone 4
Anti-trimethyl-Histone H3 (Lys4), clone MC315, monoclonal antibody (catalogue number 04-745)	EMD Millipore Corporation, Temecula (USA)	For ChIP, directed against acetylated lysine 5 of histone 4
Anti-Histone H3 antibody (catalogue number ab1791)	Abcam	For ChIP, directed against the C-terminus of histone 3
Anti-serum α-SBE A 1.4 (rabbit) PSLB 16/139 (11.04.2002)	Institute of Biology, RWTH Aachen	Negative control

2.1.5 Commercially available reaction kits

Table 2.5: Commercially available reaction kits used throughout the experiments.

Reaction Kit	Supplier	Purpose
Platinum®SYBR® Green qPCR SuperMix-UDG with Rox	Invitrogen, Carlsbad (USA)	Quantitative PCR
MSB® Spin PCRapace	STRATEG Biomedical AG, Birkenfeld	Isolation of DNA

2.1.6 Enzymes

Table 2.6: Enzymes used throughout the experiments.

Enzyme	Supplier	Purpose
DNaseI	Invitrogen, Carlsbad (USA)	DNA digestion during cDNA-synthesis
M-MLV Reverse Transcriptase	Promega, Mannheim	cDNA-synthesis

2.1.7 Size standards for the use in gel electrophoresis

Table 2.7: Size standards for the use in gel electrophoresis.

DNA Ladders	Supplier
GeneRuler™ 50 bp Ladder	Thermo Scientific, Waltham (USA)
GeneRuler™ 1 kb Ladder	Thermo Scientific, Waltham (USA)

2.1.8 Frequently used buffers and respective ingredients

Table 2.8: Frequently used buffers and respective ingredients.

Buffer	Ingredients	Final concentration
Agarose buffer (for gel electrophoresis)	Glycerol	60 % (v/v)
	EDTA, pH 8.0	60 mM
	Tris-HCl, pH 7.6	10 mM
	Orange G	0.03 % (v/v)
Buffer A	Sodium butyrate	10 mM
	Sucrose	0.4 M
	Tris, pH 8.0	10 mM
	β-Mercaptoethanol	5 mM
	PMSF	0.1 mM
	Formaldehyde	3 %
Buffer B	Sodium butyrate	10 mM

	Sucrose	0.4 M
	Tris, pH 8.0	10 mM
	β-Mercaptoethanol	5 mM
	cOmplete™ Protease Inhibitor Cocktail	1 X
	PMSF	0.1 mM
Buffer C	Sodium butyrate	10 mM
	Sucrose	0.25 M
	Tris, pH 8.0	10 mM
	β-Mercaptoethanol	5 mM
	$MgCl_2$	10 mM
	Triton X-100	1 %
	cOmplete™ Protease Inhibitor Cocktail	1 X
	PMSF	0.1 mM
Buffer D	Sodium butyrate	10 mM
	Sucrose	1.64 M
	Tris, pH 8.0	10 mM
	β-Mercaptoethanol	5 mM
	$MgCl_2$	2 mM
	Triton X-100	0.15 %
	cOmplete™ Protease Inhibitor Cocktail	1 X
	PMSF	0.1 mM
Buffer E	Tris, pH 8.0	25 mM
	EDTA, pH 8.0	5 mM
	SDS, 20 %	0.5 %
	cOmplete™ Protease Inhibitor Cocktail	1 X
	PMSF	0.1 mM
Buffer F	Tris, pH 8.0	50 mM
	EDTA, pH 8.0	1 mM
	NaCl	150 mM
	Triton X-100	0.1 %
Buffer G	Tris, pH 6.8	62.5 mM

	NaCl	200 mM
	SDS, 20 %	2 %
	Dithiothreitol (DTT)	10 mM
DNase buffer	Tris-HCl, pH 7.5	0.2 M
	$MgCl_2$	20 mM
Glycine	Glycine	2 M
M-MLV buffer	Tris-HCl, pH 8.3	50 mM
	$MgCl_2$	7 mM
	KCl	40 mM
	DTT	10 mM
	BSA	0.1 mg/ml
	(^{3}H)dTTP	0.5 mM
	oligo(dT)	0.025 mM
	poly(A)	0.25 mM
	NP-40	0.01 %
DNA extraction buffer	Tris-HCl, pH 8.0	100 mM
	NaCl	100 mM
	EDTA, pH 8.0	50 mM
	SDS, 20 %	2 %
TE buffer	Tris, pH 8.0	10 mM
	EDTA, pH 8.0	1 mM
Tris-Acetate-EDTA buffer (50x)	Tris-HCl, pH 8.0	2 M
(TAE buffer)	Glacial acetic acid	5 % (v/v)
	EDTA, pH 8.0	50 mM
Trizol	Guanidinium thiocyanate	0.8 M
	Ammonium thiocyanate	0.4 M
	Sodium acetate, pH 5.0	0.1 M
	Glycerol	5 % (v/v)
	Phenol (equilibrated)	38 % (v/v)
Wash buffer “low salt”	NaCl	150 mM
	SDS, 20 %	0.1 %
	Triton X-100	1 %
	EDTA, pH 8.0	2 mM
	Tris, pH 8.0	20 mM
Wash buffer “high salt”	NaCl	500 nM

	SDS, 20 %	0.1 %
	Triton X-100	1 %
	EDTA, pH 8.0	2 mM
	Tris, pH 8.0	20 mM
Wash buffer “lithium chloride”	LiCl	500 nM
	NP-40	0.1 %
	Sodium deoxycholate	1 %
	EDTA, pH 8.0	2 mM
	Tris, pH 8.0	20 mM

2.1.9 Oligonucleotide sequences used for qPCR reactions

The oligonucleotides used for qPCR reactions throughout the experiments, were purchased at Metabion (Martinsried) and Eurofins Genomics (Ebersberg).

Table 2.9: Oligonucleotide sequences for qPCR reactions.

Oligonucleotide	**ID**	**Sequence**	**Purpose**
C4-*PEPC* mRNA fw	96	AGAACTCAAGCCCTTTGGGAAGC	Evaluation of the
C4-*PEPC* mRNA rev	97	GtCGGCGAACTCCTTGGACAGC	C4-*PEPC* mRNA level
C4-*PEPC* -1050 fw	4271	AATACGTACATTTAAGCACACAGTCTATAT	Position -1050 bp
C4-*PEPC* -1050 rev	2321	CACTTGGCAGCGGTGAAGATAC	relative to TIS, C4-*PEPC*
C4-*PEPC* -900 fw	5714	CAAGTGCCAACAACACATCGC	Position -900 bp relative
C4-*PEPC* -900 rev	5715	GAAGGGCACCATACATATAGGG	to TIS, C4-*PEPC*
C4-*PEPC* -500 fw	1451	CTCTTAGCCACAGCCGCCTCA	Position -500 bp relative
C4-*PEPC* -500 rev	1452	CGTCTGTTAGCAAGTAGGCTGCA	to TIS, C4-*PEPC*
C4-*PEPC* -400 fw	1435	CCCTCTCCACATCCTGCAAAGC	Position -400 bp relative
C4-*PEPC* -400 rev	1436	ATTCCGTTGGCTAATTGGGTAGCA	to TIS, C4-*PEPC*
C4-*PEPC* -300 fw	1774	GCACGTCAACAGCACCGAGC	Position -300 bp relative
C4-*PEPC* -300 rev	1775	GTGGAGAGGGGTGTCTGCTAC	to TIS, C4-*PEPC*
C4-*PEPC* -200 fw	106	CGATTGCCGCCAGCAGT	Position -200 bp relative
C4-*PEPC* -200 rev	107	GAACCGGCTGTGGCTGAG	to TIS, C4-*PEPC*
C4-*PEPC* -100 fw	1441	CGGTTACCGCCGATCACATGC	Position -100 bp relative

C4-*PEPC* -100 rev	1442	CAAATAGGGATGGGGAGTCGTTGG	to TIS, C4-*PEPC*
C4-*PEPC* +10 fw	1443	AACGACTCCCCATCCCTATTTGAAC	Position +10 bp relative
C4-*PEPC* +10 rev	1444	AGCAGGGAAGCGAGACGGTTG	to TIS, C4-*PEPC*
C4-*PPDK* mRNA fw	1075	GTGGAGAACACGGTGGAGAGCC	Evaluation of the
C4-*PPDK* mRNA rev	1077	CACCAGCAGCAGGCAATCCGG	C4-*PPDK* mRNA level
C4-*PPDK* -1200 fw	1739	AGGGGTATTGTGAACAAGAGGATG	Position -1200 bp
C4-*PPDK* -1200 rev	1740	CCAATTCCTCGCAAAGACACTTCAC	relative to TIS, C4-*PPDK*
C4-*PPDK* -760 fw	2024	TGGAGGCGTTGGCTAAAGTAC	Position -760 bp relative
C4-*PPDK* -760 rev	2025	AGAGGTAAATCAGATGACTACAAAAGAAAG	to TIS, C4-*PPDK*
C4-*PPDK* -350 fw	2032	TTTCCTTAAAACCGTGCTCTATCTTT	Position -350 bp relative
C4-*PPDK* -350 rev	2033	GCACACGCGGCACGA	to TIS, C4-*PPDK*
C4-*PPDK* -150 fw	1578	CACTATAGCCACTCGCCGCAAG	Position -150 bp relative
C4-*PPDK* -150 rev	1581	CTGCTCACCTTATCCCGGACGT	to TIS, C4-*PPDK*
C4-*PPDK* +80 fw	3323	CCGTTCGCCTCTCACCTTTTCG	Position +80 bp relative
C4-*PPDK* +80 rev	3324	GCGCGTAGCTCGATGGGTTG	to TIS, C4-*PPDK*
Random nonamer primer		NNNNNNNNN	Reverse transcription

2.2 Methods

To investigate the modification state of histones, chromatin was isolated from maize and rice leaves (Bowler *et al.*, 2004; Offermann *et al.*, 2008). For this purpose, 2-week-old plants have been used. For experiments simulating nitrogen depletion, 3-week-old plants have been used (or 2-week-old plants in the case of rice). The method was based on the covalent binding of histone proteins to the associated DNA by the use of formaldehyde. This made it possible to isolate chromatin from the formaldehyde-treated leaf-material. During the subsequent chromatin immunoprecipitation (ChIP) the modified histones were concentrated by the use of protein A agarose and antibodies directed against the modifications at question. The degree at which the modification was observed at a specific location throughout the gene was measured via qPCR. Concerning the crosslinking of the samples, adaptations of the experimental procedure (Bowler *et al.*, 2004; Offermann *et al.*, 2008) were necessary, depending on whether maize or rice was used (2.2.3).

2.2.1 Plant cultivation

The following plant cultivars and transgenic lines were used for the experiments:

Table 2.10: Maize and rice lines used as experimental plants.

Species	Line	Manufacturer designation	Supplier
Zea mays	L. cv. Montello	Montello	agaSAAT GmbH, Neukirchen-Vluyn
Oryza sativa indica	IR64	IR64_A009, wild-type rice	IRRI, Los Baños (Philippines)
Oryza sativa indica	R1001	IR64-IRS522/485-028 (expressing C4-*PEPC*)	IRRI, Los Baños (Philippines)
Oryza sativa indica	R1002	IR64-IRS522/485-060 (expressing C4-*PEPC*)	IRRI, Los Baños (Philippines)
Oryza sativa indica	R1007	IR64-IRS609/485-002 (expressing C4-*PPDK*)	IRRI, Los Baños (Philippines)
Oryza sativa indica	R1008	IR64-IRS609/485-011 (expressing C4-*PPDK*)	IRRI, Los Baños (Philippines)

The experiments were performed with maize as control and two different transgenic rice lines for each gene. The transgenic rice was derived from the *Oryza sativa indica* rice IR64, produced by the International Rice Research Institute (IRRI). The plants were used to investigate the histone code of C4 genes that are expressed in rice. To produce the transgenic rice, *Oryza sativa indica* IR64 was transformed by the IRRI with two independent constructs, one construct containing the full length maize C4-*PEPC* (the inserted coding sequence comprises 6.6 kb) or C4-*PPDK* (the inserted coding sequence comprises 7.3 kb) gene as well as the corresponding maize promoter of C4-*PEPC* or C4-*PPDK,* and another construct containing a CaMV35S promoter driven hygromycin selection marker. For the transformation with C4-genes, the pSC0 vector (GenBank accession number KT365905) was used, which was originally created from the pMDC123 vector (Curtis and Grossniklaus, 2003). For hygromycin resistance, the pCAMBIA1300 vector was used (GenBank accession number AF234296.1). The sequences are based on GenBank accession numbers GRMZM2G083841/T01 for C4-*PEPC* and GRMZM2G306345/T05 for C4-*PPDK*. The upstream region of C4-*PEPC* comprises 1208 bp in transgenic rice, while the upstream region of C4-*PPDK* comprises 1300 bp in transgenic rice. For this purpose, embryo tissue cultures of IR64 *Oryza sativa indica* rice were co-transformed with *Agrobacterium* lines of pSC0 (containing the respective C4-gene) and pCAMBIA1300. Selection was based on hygromycin resistance. Finally it was screened for the respective gene by PCR.

Maize control plants were cultivated in Einheitserde VM soil in a CLF Plant Climatics GroBank. A diurnal rhythm of 16 h light and 8 h darkness was applied with temperatures of 25 °C during the light period and 20 °C during the dark period. Photon flux density for maize was about 140 µmol m^2 s^1. Rice was cultivated in Einheitserde “Topferde CLT”. Rice seeds were incubated at 50 °C for five days and transferred to tap water afterwards, for another seven days. Seeds in tap water were kept in a CLF Plant Climatics GroBank at the same conditions at which plants already transferred to soil were kept. After this time period, seeds germinated and could be transferred to soil. A diurnal rhythm of 16 h of light and 8 h of darkness was applied, with a temperature of 25 °C during the light phase and 20 °C during the dark phase. Photon flux density was about 140 µmol m^2 s^1. Depending on the experiment, maize and rice plants were grown for two to three weeks before harvest and treatment.

2.2.2 Simulation of different environmental conditions and influences

2.2.2.1 Light induction

The reaction of C4-*PEPC* and C4-*PPDK* in transgenic rice to light exposure was tested on the chromatin level and with regard to the mRNA abundance of C4-*PEPC* and C4-*PPDK*. To investigate the effect of light and darkness, the material was crosslinked and chromatin was isolated after 48 h of darkness, after 4 h of light influence for C4-*PEPC*, or after 1 h of light influence for C4-*PPDK,* respectively. The plants were crosslinked with 1 % of formaldehyde in the case of rice, or 3 % of formaldehyde in the case of maize, respectively and stored at -80 °C. Additionally, a portion of the material was frozen in liquid nitrogen before crosslinking and used for RNA isolation.

2.2.2.2 Diurnal regulation

In maize, *PEPC* is known to show a specific expression level, depending on the observed time point. Therefore, it was tested, whether this diurnal regulation could also be observed in transgenic rice, expressing C4-*PEPC*. For this purpose the mRNA level and the abundance of the investigated histone modifications was measured and compared in maize and transgenic rice. The leaves were harvested and crosslinked with 1 % or 3 % of formaldehyde respectively after 4 h of illumination, after 16 h of illumination, or after 48 h of darkness and stored at -80 °C. Additionally, a portion of the material was frozen in liquid nitrogen before crosslinking and used for RNA isolation.

2.2.2.3 Nitrogen depletion

The reaction of transgenic rice to stress caused by nitrogen depletion was tested on the chromatin level and with regard to the mRNA abundance of *PEPC*. To provoke stress and nitrogen depletion, the plants were cut 1 h after the onset of light and the leaves were incubated in tap water for further 3 h. Afterwards a crosslink with formaldehyde was performed (2.2.3). As a comparison, intact plants were harvested and crosslinked after 4 h of illumination. In parallel, plants were incubated in a 0.5 µM

zeatin solution, containing KNO_3, instead of tap water 1 h after the onset of light and incubated for further 3 h.

For the treatment simulating nitrogen depletion, 17 to 18 days old maize and 14 days old rice were cut right above the soil. To prevent air from entering the leaves due to the cut, the plants were transferred to tap water and the leaves were cut off under water. They were placed in tap water or the 0.5 µM zeatin solution respectively. The intact reference plants were not treated but kept under normal conditions before being harvested after 4 h of exposure to light. After the incubation, the plants were crosslinked with 1 % or 3 % of formaldehyde, respectively, and stored at -80 °C. Additionally, a portion of the material was frozen in liquid nitrogen before crosslinking and used for RNA isolation.

2.2.3 Isolation of chromatin from maize and rice leaves

For the isolation of chromatin from maize plants, 3 g leaf material of maize were cut into pieces of approximately 1 cm length and transferred to 200 ml of Buffer A (Table 2.8). The material was vacuum infiltrated with the buffer under stirring at 50 mbar for 2.5 min. During this time the vacuum was interrupted six to seven times. Then the leaf material was incubated in Buffer A for additional 2.5 min under stirring, without vacuum. To stop the crosslinking reaction caused by the formaldehyde in Buffer A, 20 ml of 2 M glycine were added and the leaf material was vacuum-infiltrated for 5 min under constant stirring. The vacuum was interrupted every 30 s. The leaf material was rinsed thoroughly under VE-H_2O and dried. It was then frozen in liquid nitrogen and grinded with a cooled mortar, to get a fine powder. The material was stored at -80°C until it was used for the chromatin immunoprecipitation.

Alternatively, 0.75 g leaf material of rice were cut into pieces of approximately 1 cm length and transferred to 200 ml of Buffer A. The material was vacuum infiltrated with the Buffer A, containing 1 % of formaldehyde, under stirring, at 50 mbar for 4 min. The vacuum was interrupted six to seven times and the leaf material was incubated in the buffer for an additional 1 min under stirring but without vacuum. Then 20 ml of 2 M glycine were added. From this point on, the protocol corresponded to the protocol described above for maize plants.

The powder gained from the crosslinked leaf material was resuspended and incubated in 40 ml of Buffer B for 15 min at 4 °C under constant shaking. To remove cell debris, the solution was filtrated through four layers of miracloth. The filtrate was centrifuged at 4 °C for 20 min at 2880 x g. The

supernatant was discarded. The remaining pellet was resuspended in 1 ml of Buffer C. The solution was then centrifuged at 12000 x g at 4 °C for 10 min. The supernatant was discarded. The pellet was resuspended in 300 µl Buffer D, used to overlay 1.5 ml of Buffer D and then centrifuged at 16000 x g at 4 °C for 1 h. In the next step the chromatin was sheared in a cooled ultrasonic bath, to break it down into fragments. For this purpose, the supernatant was discarded and the pellet resuspended in 300 µl of Buffer E. A 20 µl aliquot (or 40 µl in the case of rice) was taken to evaluate the quality and degree of the shearing step. The samples were sonicated in the pre-cooled water bath of a bioruptor. The chosen setting was “high”, with intervals 30/30 for 10 min. After sonication, the samples were centrifuged for 5 min at 16000 x g and 4 °C. The supernatant was transferred to a new 1.5 ml reaction tube. Another 20 µl aliquot (or a 40 µl aliquot for rice, respectively) was taken for the control of the shearing quality (which was subsequently compared to the aliquot taken before the shearing step). The supernatant was then further diluted with another 300 µl of Buffer E. The samples were frozen in liquid nitrogen and stored at -80°C until they were used for the antibody treatment.

The quality of the isolated chromatin was tested by applying the 20 µl and 40 µl aliquots of sheared and unsheared chromatin to a 2 % agarose gel. For this purpose, the formaldehyde-mediated crosslink was reversed by adding 100 µl of Buffer G to the samples. They were incubated in Buffer G under constant shaking for 5 min at room temperature, after which they were transferred to 65 °C over night. Afterwards the DNA was isolated with the MSB® Spin PCRapace kit (Stratec molecular).

In the next step, 400 µl of Binding Buffer were added to each sample before it was incubated under shaking for 5 min at room temperature. Afterwards the samples were centrifuged for 5 min at room temperature and 500 x g. The supernatant was transferred to a spin column, provided by the supplier. A centrifugation step at 500 x g at room temperature was performed for 5 min. The flowthrough was discarded. Then 500 µl of Binding Buffer were added to the column and another centrifugation step for 3 min at 500 x g and room temperature followed. Again, the flowthrough was discarded. The samples were again centrifuged for 3 min at 500 x g at room temperature. The column was transferred to a new 1.5 ml reaction tube. Finally 20 µl of the provided Elution Buffer were added to each sample. After an incubation time of 10 min at room temperature, the samples were centrifuged at 4500 x g at room temperature for 5 min. During this step the DNA was eluted and collected in the reaction tube.

2.2.4 Agarose gel electrophoresis

For a control of the shearing quality, the DNA was applied to an agarose gel for gel electrophoresis. The DNA was blended with 5 µl of Loading Dye for subsequent gel electrophoresis on a 2 % agarose gel. The gel was run for 45 to 60 min at 120 V. The electrophoresis took place in a 1 x TAE buffer (Table 2.8) with 0.25 µg ethidiumbromide per ml. For a better detection, the gel itself was also prepared with 0.25 µg of ethidiumbromide per ml. The size of the isolated DNA fragments was determined by comparison with a 1 kb DNA ladder (Table 2.7).

2.2.5 Chromatin immunoprecipitation

The isolated chromatin was used for immunoprecipitation with antibodies directed against specific modifications or the unmodified H3C-terminus of histones respectively and protein A agarose beads.

The histone modifications investigated by the use of this method were H3K9ac, H4K5ac, H3K4me3 and H3K18ac. Apart from the antibodies directed against these modifications, an antibody directed against the invariant C-terminal end of histone H3 was used to evaluate the nucleosome density. The nucleosome density was afterwards used as reference, to evaluate the ratio between nucleosomes in general to specific modifications. As negative control and to remove background noise, the approach was also performed using an unspecific antibody.

Before the ChIP was performed, the chromatin was precleared to prevent unspecific interactions. For this purpose 200 µl of chromatin were added to 1.8 ml of Buffer F and 40 µl of protein A agarose. The sample was then incubated at 4 °C under constant shaking on an overhead shaker for 1 h. Afterwards the protein A agarose beads were separated from the supernatant by 5 min of centrifugation at 4 °C and 500 x g. The supernatant was collected while the beads were discarded. The precleared supernatant was then used for the actual ChIP directly, or stored at -80 °C for later use.

For each antibody directed against one of the histone modifications, as well as for the unspecific antibody and the antibody directed against the invariant C-terminus of histone 3, 400 µl of precleared chromatin were transferred to 1.5 ml reaction tubes. For the measurement of the overall DNA amount, an additional 40 µl aliquot was prepared. It was stored at 4 °C and not used for the ChIP.

Table 2.11: Samples subjected to Chromatin Immunoprecipitation.

Volume of precleared chromatin (µl)	Antibody for ChIP	Volume of the Antibody (µl)
400	H3K9ac	5
400	H4K5ac	5
400	H3K18ac	5
400	H3K4me3	2.5
400	H3C term (invariant C-terminus of H3)	1
400	Serum α-SBE A 1.4 (unspecific negative control)	5
40	Reference/input for total DNA	

To each sample 30 µl of protein A agarose were added, as well as the respective antibody in the specific amount (Table 2.11). The samples were incubated under constant shaking on the overhead shaker at 4 °C over night.

After the precipitation, the protein A agarose beads, which were now bound to the antibodies and the corresponding chromatin, were washed with a series of different washing buffers. For this purpose the samples were centrifuged for 5 min at 4 °C and 500 x g. The supernatant was discarded and the washing buffers were applied in the following order:

Table 2.12: Washing of Protein A agarose beads after chromatin Immunoprecipitation.

Washing step	Buffer	Applied volume (µl)	Incubation time (min)
1	Wash buffer “low salt”	900	10
2	Wash buffer “high salt”	900	10
3	Wash buffer “lithium chloride”	900	10
4	TE buffer	900	10
5	TE buffer	900	10

Each of the five washing steps was performed by adding 900 µl of the corresponding washing buffer to the protein A agarose beads. The samples were then incubated for 10 min on the overhead shaker

at 4 °C under constant shaking. Afterwards they were centrifuged for 5 min at 4 °C and 500 x g. The supernatant was discarded and 900 µl of the next washing buffer were applied. After the last washing step, the supernatant was removed completely and the samples were treated with 100 µl of Buffer G each. They were incubated under constant shaking for 5 min at room temperature, after which they were transferred to 65 °C over night, to reverse the crosslink.

For the subsequent DNA isolation the MSB® Spin PCRapace kit (Stratec molecular) was used, as described in detail in 2.2.3. Again, the isolation was performed according to the manufacturers manual, but with 80 µl of Elution Buffer instead of 20 µl, as in 2.2.3. The DNA was stored at -20 °C for subsequent use in qPCR reactions.

2.2.6 RNA isolation

RNA was isolated from 40 mg of grinded leaf material. For this purpose 1 ml of Trizol (Table 2.8) was added to the powder, which was then placed on a shaker for 15 min at room temperature. Afterwards 200 µl of chloroform were added and the samples were incubated for another 10 min under constant shaking at room temperature. The samples were then centrifuged for 15 min at 4 °C and 16000 x g for a first separation of phases. Another 400 µl of chloroform were added to the separated aqueous phase and the samples were incubated under constant shaking for 10 min at room temperature. Then they were centrifuged for 10 min at 4 °C and 16000 x g. Finally 250 µl of the upper, aqueous phase were transferred to a new 1.5 ml reaction tube and 500 µl of cold 99 % ethanol were added. The samples were inverted several times and then stored at -20 °C over night.

The next day, the sample was centrifuged at 4 °C and 16000 x g for 15 min. The supernatant was removed and the pellet was washed with 200 µl of 70 % ethanol. The ethanol was removed. When no further ethanol residues were visible in the tubes, the RNA pellet was resolved in 20 µl of VE-H_2O.

The concentration of the isolated RNA was measured applying a dual approach with a BioTek SYNERGY Mx Microplate Reader, using the BioTek Gen5 Take3 Module and the Take3™ Multivolume Plate using the Gen5™ 1.11 software. The measurements took place at the wavelengths 260 nm and 280 nm. As quality control the 260 nm to 280 nm absorption ratio was calculated, indicating the purity of RNA. For an additional control of the RNA quality, it was possible to apply the samples to a 2 % agarose gel and perform a gel electrophoresis, as described in 1.1.1. Here, 7 µl of RNA were blended with 3 µl of Loading Dye before being applied to the gel.

2.2.7 cDNA synthesis

A total of 100 ng of RNA were added to each approach.

Table 2.13: Components and respective concentrations/volumes for the DNaseI digestion reaction mixture.

Component	Concentration/Volume
DNaseI	1.2 U
DNaseI buffer	1.2 x
RNA	100 ng
H_2O	Ad 12 µl

Table 2.14: Components and respective concentrations/volumes for the reverse transcription reaction mixture.

Component	Concentration/Volume
dNTP mixture	1 mM
M-MLV buffer	1 x
M-MLV reverse transcriptase	200 U
H_2O	Ad 7 µl

Prior to the cDNA synthesis, the samples were subjected to a DNaseI digestion to remove contaminations with genomic DNA. For each sample 1.2 µl DNaseI (according to 1.2 U) were mixed with 1.2 µl of 10 x DNase reaction buffer, 7.6 µl VE-H_2O and 2 µl of RNA. The digestion was performed at 37 °C for 30 min. The enzyme was then inactivated at 70 °C for 15 min. Afterwards the samples were cooled immediately. Then 1 µl of random nonamer primer (50µM) (2.1.9) was added to each sample. The samples were incubated at 70 °C for another 5 min. Afterwards they were stored on ice and the reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase, M-MLV-RT) was added. For this purpose two different mixes were prepared, one containing 2 µl of dNTPs, 4 µl of RT-buffer (5x), 0.5 µl of VE-H_2O and 0.5 µl of M-MLV reverse transcriptase for each sample (referred to as "+RT", indicating the presence of reverse transcriptase). The other one did not contain reverse transcriptase, but an equal amount of VE-H_2O (referred to as "-RT", indicating the absence of reverse transcriptase). The -RT sample was used as a control for the DNaseI digestion and was applied in a parallel approach with a representative selection of samples. The synthesis took place at 37 °C in a water bath for 1 h. Afterwards the samples were incubated at 70 °C for further 10 min to stop the reaction. They were then stored at -20 °C for subsequent qPCR measurements.

2.2.8 Quantitative polymerase chain reaction

The qPCR was performed with the Platinum® SYBR® Green qPCR SuperMix-UDG and signals were detected with a CCD camera. The qPCR conditions are shown in (Table 2.15).

Table 2.15: qPCR run method with thermoprofile.

Stage	Step	Temperature (°C)	Duration	Purpose
Holding stage	1	50	2 min	Carry over protection
	2	95	2 min	Denaturation
Cycling stage (40x)	1	95	15 s	Denaturation
	2	60	1 min	Annealing and elongation
Melt curve stage	1	95	15 s	Denaturation
	2	60	1 min	Base for increase
	3	60-95/+0.3	15 s	Melting curve

The actual qPCR was followed by a Melt Curve Stage, at which the temperature was evenly increased from 60 °C to 95 °C.

To quantify the amount of DNA, the samples were compared to a standard curve based on stepwise diluted DNA. The calculated quantity of DNA was used to define ratios between the samples that had to be compared.

2.3 Mathematical methods

2.3.1 Arithmetic mean

The arithmetic mean describes the sum of a series of values divided by the number of these values. The calculation results in a value for which the sum of negative and positive deviations is equal.

$$x_{arithm} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

Formula 2.1: Arithmetic mean. x_{arithm}= arithmetic mean, n= number of values, x= value of the random sample i, i= random sample.

2.3.2 Standard deviation

By calculating the standard deviation, it is possible to compare measured values, for example of different replicates within an experimental setup. This is achieved by comparing the scattering of measured values around a given mean. The standard deviation is, therefore, an indicator of reproducibility.

$$SD = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(x_i - x_{arithm})^2}$$

Formula 2.2: Standard deviation. *SD*=standard deviation, x_{arithm}= arithmetic mean, n= number of values, x= value of the random sample i, i= random sample.

2.3.3 Percent of input

Measurements for a specific histone modification can be evaluated by referring to the total amount of DNA. This is done by the calculation of the % of input, in which the input is the total amount of DNA. For this purpose, the measured value for the modification at question is divided by the measured value for the total DNA.

2.3.4 Signal per nucleosome

Apart from the evaluation of measurements for specific modifications by referring to the total amount of DNA, it is also possible to refer to the nucleosome density instead. In this case, the measurement for the modification is compared to the nucleosome density at a specific position. For this purpose, the measured value for the modification is divided by the measured value for the nucleosome density. As described in 2.2.5, antibodies directed against the invariant C-terminal end of histone 3 are used for the ChIP, on the base of which the nucleosome density is calculated.

3 Results

In the present thesis, the influence of the external stimuli illumination and nitrogen depletion and the presence or absence of an internal diurnal rhythm on the mRNA level and histone modifications of photosynthetic C4-genes from maize, heterologously expressed in transgenic rice was evaluated. For this evaluation, it was necessary to compare the results from transgenic rice with data already known from maize. Therefore, information on the transcriptional activity and on the occurrence of histone modifications of C4-*PEPC* and C4-*PPDK,* which have been described in former studies, have been integrated in the present description of the results. Instead of only referring to them in the context of the discussion, including these data in the results is thought to provide the basis for a better comparison between the situation in the donor species maize and the recipient species rice. The transgenic rice lines which have been used for the experiments were provided by the International Rice Research Institute. In the present thesis, these lines were designated as R1001 and R1002 for those which were expressing C4-*PEPC*. The designations for the rice lines expressing C4-*PPDK* were R1007 and R1008. In the experiments that were performed to investigate the influence of illumination and prolonged darkness on transgenic rice expressing C4-genes, both of the respective rice lines were used in each case. The mRNA level reacted similar to external stimuli in different lineages and was, therefore, thought to be independent of the exact integration site. Therefore, only R1001 was used for experiments concentrating on the influence of nitrogen depletion and the internal diurnal rhythm on C4-*PEPC*.

3.1 The influence of illumination or darkness on the mRNA level and chromatin state of C4-*PEPC* in maize and transgenic rice

Light strongly induces the transcriptional activity of C4-*PEPC* (Sheen, 1999; Danker *et al.*, 2008; Offermann *et al.*, 2008). The light inducibility of C4-*PEPC* was also part of the present thesis. Here, the focus lay on the ability of the transgenic rice lines R1001 and R1002, which are expressing maize C4-*PEPC*, to regulate the gene in the same way, in which it would be regulated in the donor species maize.

To investigate the regulation of C4-*PEPC* in transgenic rice with regard to the illumination status, R1001 and R1002 were harvested after 4 h of illumination and after 48 h of prolonged darkness. Maize was used as a control. It was treated and harvested according to the procedure for transgenic rice. The plant material was either used for RNA isolation (2.2.6), followed by subsequent cDNA-synthesis (2.2.7), to evaluate the mRNA level of C4-*PEPC*, or it was used for crosslinking experiments (2.2.3) with subsequent ChIP (2.2.5) to evaluate the chromatin status with regard to the influence of illumination or prolonged darkness. The results from the reaction of C4-*PEPC* to illumination are presented in Figure 3.1. To evaluate the chromatin state, the nucleosome density was calculated as percent (%) of input. The histone modifications H3K9ac (Figure 3.1 C), H4K5ac (Figure 3.1 E), H3K18ac (Figure 3.1 D) and H3K4me3 (Figure 3.1 F) were calculated with regard to the nucleosome density. The profile ranged from the distal promoter at position -1050 bp on the upstream promoter, to position +10 bp in the transcribed region of C4-*PEPC* (Figure 3.1 B). For a more detailed comparison, three representative positions (for the distal promoter at position -1050 bp, for the proximal promoter at position -200 bp and for the beginning of the transcribed region at position +10 bp) are shown for the control plant *Zea mays* as well as for the transgenic rice.

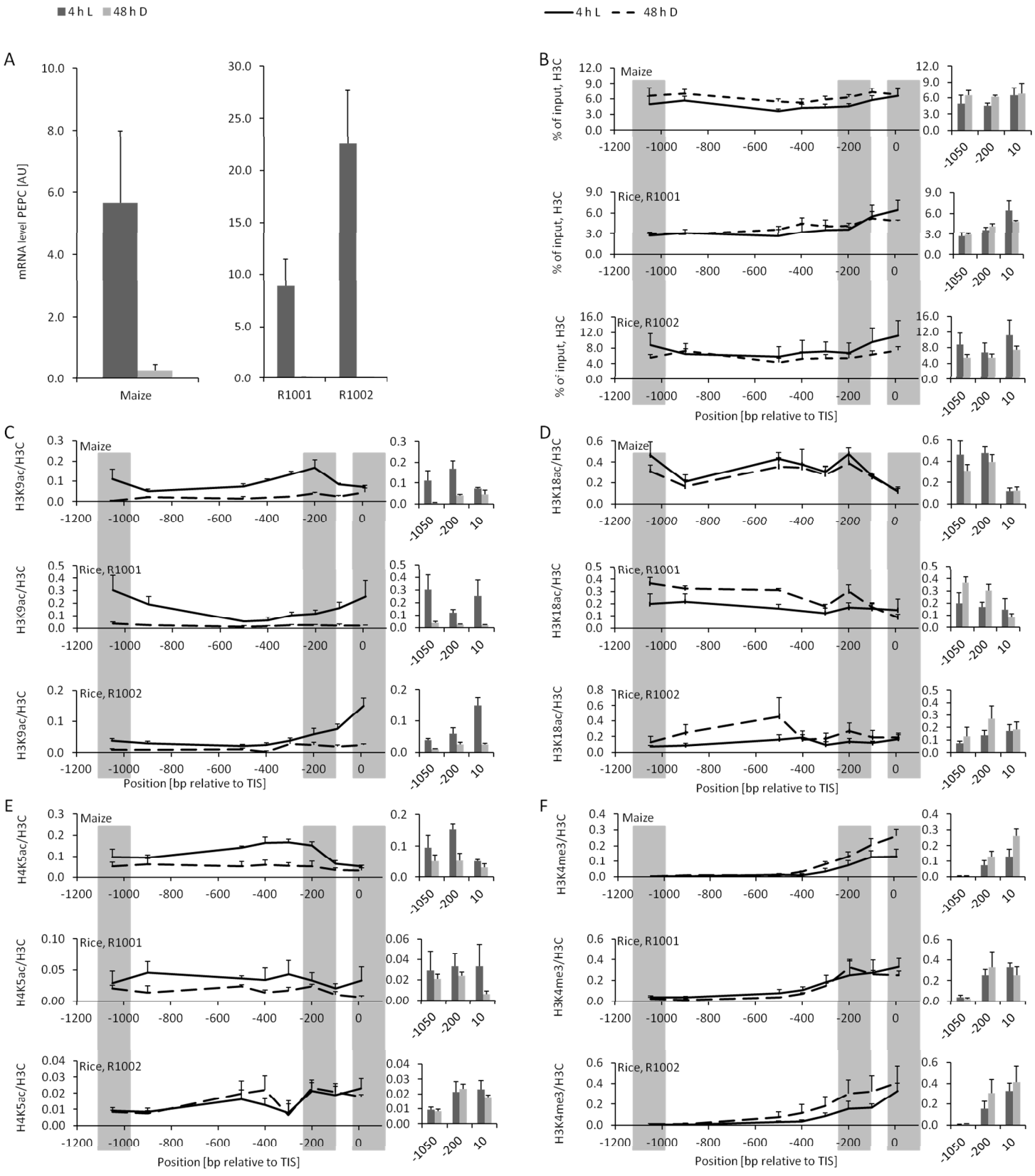
4 h L
48 h D
A
mRNA level PEPC [AU]
Maize
R1001
R1002
B
% of input, H3C
Maize
Rice, R1001
Rice, R1002
Position [bp relative to TIS]
C
H3K9ac/H3C
Maize
Rice, R1001
Rice, R1002
Position [bp relative to TIS]
D
H3K18ac/H3C
Maize
Rice, R1001
Rice, R1002
Position [bp relative to TIS]
E
H4K5ac/H3C
Maize
Rice, R1001
Rice, R1002
Position [bp relative to TIS]
F
H3K4me3/H3C
Maize
Rice, R1001
Rice, R1002
Position [bp relative to TIS]

Figure 3.1: mRNA and histone modification levels of C4-*PEPC* in maize and transgenic rice under the influence of illumination. Chromatin was precipitated with antibodies directed against the acetylations at histone 3, lysines 9 and 18 (H3K9ac and H3K18ac), histone 4, lysine 5 (H4K5ac), and the trimethylation at histone 3 lysine 4 (H3K4me3). To evaluate the nucleosome density, an antibody against the invariant C-terminal end of histone 3 (H3C) has been used. For the nucleosome density, the amount is presented as percent (%) of input. The modifications were standardized on the nucleosome density. The results refer to plants harvested after 4 h of illumination (depicted as "4 h L") and plants that were kept in darkness for 48 h before they were used for crosslinking and subsequent ChIP (depicted as "48 h D"). (A) mRNA levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1001 and R1002. (B) H3C levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1001 and R1002. (C) H3K9ac levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1001 and R1002. (D) H3K18ac levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1001 and R1002. (E) H4K5ac levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice linelines R1001 and R1002. (F) H3K4me3 levels of C4-*PEPC* in illuminated and darkened maize and the illuminated and darkened transgenic rice linelines R1001 and R1002. Data points are based on three independent experiments for ChIP experiments and the mRNA level of maize, and two independent experiments for the mRNA levels of transgenic rice. Vertical lines indicate standard errors.

3.1.1 The mRNA level of C4-*PEPC* in maize and transgenic rice under the influence of illumination or darkness

Concerning the C4-*PEPC* mRNA levels in illuminated and darkened plants of maize and transgenic rice, expressing C4-*PEPC*, a similar effect was observed in both species. Both maize and transgenic rice demonstrated a reduction of more than 90 % of mRNA levels in plants under prolonged darkness. In maize, the mRNA level was reduced by 92 % when plants were kept in darkness for 48 h (Figure 3.1 A, left). In R1001, the reduction accounted for 97 % of mRNA levels in illuminated plants, while mRNA levels of C4-*PEPC* in R1002 were reduced by 98 %, when compared to illuminated plants. In this context, it could also be observed that mRNA levels in R1002 were higher than in R1001 (Figure 3.1 A, right).

3.1.2 The nucleosome density of C4-*PEPC* in maize and transgenic rice under the influence of illumination or darkness

To compare the chromatin state in maize and transgenic rice, a profile of the nucleosome density has been recorded. The distribution of H3C in maize control plants corresponded to the data already described by Offermann *et al.* (2008). Cited study revealed an even distribution of H3C in intact control plants of maize for the part of the gene observed in the present thesis, whereby the nucleosome density was slightly increased at the distal promoter and at the beginning of the transcribed region, in comparison to the nucleosome density at the proximal promoter. In Offermann *et al.* (2008), H3C signals did not show a strong reaction to illumination. Nevertheless, a characteristic

feature of H3C distribution in maize was a 3-fold increase at the beginning of the transcribed region (Offermann *et al.*, 2008).

In the control plant maize, the distribution of H3C, dependent on the position, was insofar identical in illuminated and darkened plants, as both were showing maximal values at the distal promoter and at the beginning of the transcribed region, while the nucleosome density was lowest at the proximal promoter (Figure 3.1 B). The H3C levels did not change upon illumination or prolonged darkness, therefore, the results corresponded to previously known data (Offermann *et al.*, 2008). With regard to R1001 it was observed that the nucleosome density showed a similar distribution over the observed part of the gene, as the control plant maize did. Especially at the proximal promoter and the beginning of the transcribed region, similarities were observed. For darkened plants of R1001 the distribution of the nucleosome density was similar to the distribution in illuminated plants. Nucleosome density on C4-*PEPC* in darkened R1001 was lowest at position -1050 bp of the distal promoter. It then increased towards the proximal promoter. Similar to the situation in the donor species maize, the observed nucleosome density in transgenic rice did not show differences between illuminated and darkened plants. For R1002, the second transgenic rice line expressing C4-*PEPC*, the course of the nucleosome density over the observed region of the gene was also similar to the results obtained in maize. The distribution of H3C signals corresponded to the results observed for maize and R1001. Apart from this, darkened plants of R1002 did not show increased nucleosome density upon illumination or prolonged darkness. (Figure 3.1 B).

Summarizing, the nucleosome density on C4-*PEPC* showed a similar distribution over the observed part of the gene in maize and transgenic rice, independent of the integration site. The reaction of the nucleosome density towards the external stimulus light was also similar in both species. There was no increase in nucleosome density if plants were kept in complete darkness for 48 h, neither in maize, nor in rice. Nevertheless, both species demonstrated reduced mRNA levels, if the plants were kept in darkness.

3.1.3 The H3K9ac level of C4-*PEPC* in maize and transgenic rice under the influence of illumination or pdarkness

In previous studies, a 3 to 10-fold light induction of H3K9ac (depending on the position) has been demonstrated for maize, whereby maximal values were measured on the promoter. In this context, a peak at the distal promoter was identified at position -2000 bp relative to the TIS (Offermann *et al.*,

2008). In the present thesis, it was not possible to evaluate positions farther upstream than -1050 bp relative to the TIS, due to the limitations caused by the C4-*PEPC* construct introduced into rice. Nevertheless, the position -1050 bp relative to the TIS was used in the present thesis to evaluate the reaction of the distal promoter to illumination. The promoter in general comprises a distal portion beginning at position -600 bp relative to the TIS and a proximal portion spanning the region between the TIS and position -600 bp relative to the TIS. Position -1050 bp relative to the TIS, which has been measured in the present thesis is, therefore, suitable to draw conclusions concerning the distal promoter. Nevertheless, if it is compared to the results from the distal promoter at position -2000 bp relative to the TIS, presented in Offermann *et al.* (2008), it can only indicate a tendency, which is not as pronounced as the reaction at position -2000 bp. The density of the histone modification has been evaluated in reference to the nucleosome density at a given position.

Concerning the control plant maize, illuminated plants showed increased H3K9ac levels at positions -1050 bp and -200 bp relative to the TIS, which means at the distal promoter and at the proximal promoter. In between, a minimum was measured at position -900 bp relative to the TIS. H3K9ac levels dropped again between the increased value at position -200 bp and the beginning of the transcribed region, which was measured at position +10 bp relative to the TIS (Figure 3.1 C, right).

When R1001 was compared to the control species maize, similarities in the modifications distribution were observed. At the distal promoter, illuminated plants of R1001 demonstrated increased H3K9ac levels, as did maize. In addition to this, the peak at position -200 bp, which was observed in maize, could also be detected in transgenic rice, in which it was shifted towards the beginning of the transcribed region. In transgenic rice, the peak was observed at position +10 bp relative to the TIS. Lowest acetylation levels for R1001 were measured at position -500 bp relative to the TIS (Figure 3.1 C). R1002, showed a similar distribution of H3K9ac as R1001. In contrast to R1001, the increase at position -1050 bp relative to the TIS could not be observed. Therefore, R1002 differed from R1001 and maize with regard to acetylation events at the distal promoter. Similar to R1001, R1002 showed an H3K9ac minimum at position -500 bp relative to the TIS. This acetylation minimum was followed by a constant increase towards the beginning of the transcribed region, reaching maximal acetylation values at position +10 bp relative to the TIS (Figure 3.1 C). This peak corresponded to the acetylation peak at position -200 bp in the control species maize and to the acetylation peak at position +10 bp in R1001. In comparison to the acetylation peak at the proximal promoter in maize, it was shifted towards the beginning of the transcribed region.

When maize was kept in prolonged darkness, H3K9ac levels decreased at all measured positions. The peaks at positions -1050 bp and -200 bp relative to the TIS were not observed, so that H3K9ac levels remained more constant. It was no longer possible to observe positions with increased H3K9ac levels

throughout the observed part of the promoter. When R1001 plants were kept in prolonged darkness, it was possible to observe the same effect as in the control species maize. Like in maize, acetylation levels were reduced and all of the peaks that were measured in illuminated plants were not observed in darkened plants. When plants of R1002 were kept in prolonged darkness, acetylation levels were also generally reduced and the peaks disappeared, similar to the situation in R1001 and maize. Altogether, darkness led to the same effect on acetylation levels of H3K9 in both species.

Summarizing, it was observed that with regard to the distribution of H3K9ac, maize and transgenic rice showed similarities in the acetylation levels reaction towards illumination and prolonged darkness. The peak at the proximal promoter in maize was shifted in rice. It was, therefore, located at the beginning of the transcribed region. In addition to the similar distribution over the observed part of the gene, the acetylation levels reaction towards illumination or darkness was also similar in both species. Notably, the shift of the peak at the proximal promoter in maize did not lead to differences in the reaction of the mRNA level to illumination or prolonged darkness in transgenic rice. Independent of the exact position of the acetylation peak in illuminated samples of maize and transgenic rice, there was an increase of the mRNA level under the influence of light. If the plants were kept in darkness for 48 h, the mRNA level was reduced in both species to a similar extend. Therefore, the acetylation levels at all tested positions correlated with the mRNA level in both species.

3.1.4 The H4K5ac level of C4-*PEPC* in maize and transgenic rice under the influence of illumination or darkness

Similar to H3K9ac, previous studies demonstrated a 3 to 10-fold light induction for H4K5ac in maize depending on the respective position. As for H3K9ac, maximal values were measured on the promoter. In contrast to H3K9ac, the acetylation levels of H4K5ac were distributed more evenly. A peak was observed at the proximal promoter, while H4K5ac levels around position -1000 bp relative to the TIS were lower. Another peak was identified at the beginning of the transcribed region (Offermann *et al.*, 2008). The results of the present thesis in general corresponded to the data already known for H4K5ac in maize. Nevertheless, the peak at the beginning of the transcribed region could not be detected.

H4K5ac demonstrated a broad distribution in maize, with highest levels at the proximal promoter, between positions -400 bp relative to the TIS and -200 bp relative to the TIS. Between the proximal

promoter and the beginning of the transcribed region, the acetylation level of H4K5 decreased again, reaching lowest levels at the beginning of the transcribed region (position +10 bp relative to the TIS) (Figure 3.1 E, left). R1001 demonstrated a similar distribution of H4K5ac over the observed part of the gene. Although the distribution was broad in both maize and R1001, H4K5ac was distributed more evenly between the distal and proximal promoter region in R1001. Notably, the peak at the proximal promoter in the donor species maize could not be detected in R1001. The acetylation level of H4K5 in R1002 was in general low and the peak observed at the proximal promoter in maize was absent in R1002 (Figure 3.1 E, left).

When maize was kept in prolonged darkness, H4K5ac levels decreased at all positions, as did H3K9ac levels. The peak at positions -400 bp to -200 bp was not observed. H4K5ac levels in darkened maize demonstrated an even distribution without regions representing increased H4K5ac levels. This effect could also be observed in R1001. Concerning the modifications reaction towards illumination, it was again possible to observe similarities between maize and R1001, while the situation in R1002 differed. For all tested positions, maize and R1001 demonstrated decreasing H4K5ac levels if the plants were subjected to prolonged darkness.

Summarizing, the distribution of H4K5ac over the observed part of the gene, as well as the acetylations reaction towards illumination showed similarities between maize and transgenic rice. The distribution of H4K5 acetylation differed strongly in the two transgenic rice lines R1001 and R1002. In general, it has to be considered that the acetylation levels for H4K5ac were lower in transgenic rice, than in maize. The strong light induction which was observed for maize at the proximal promoter was not detected in R1001 to this extend. For R1002 it was not possible to detect light induction of H4K5ac at all. However, this did not provoke any differences concerning the mRNA levels reaction towards illumination or darkness in maize and transgenic rice. Therefore, for R1001, a weak correlation between the mRNA level and the acetylation level was observed. This demonstrated a similarity between maize and R1001. For R1002 on the other hand, it was not possible to observe a correlation between the mRNA level and the acetylation level of H4K5.

3.1.5 The H3K18ac level of C4-*PEPC* in maize and transgenic rice under the influence of illumination or darkness

Additionally, a profile of the H3K18ac distribution over the observed part of C4-*PEPC* has been recorded for maize and transgenic rice (Figure 3.1 D).

Previous data presented by Offermann *et al.* (2008) demonstrated reduced levels of H3K18ac behind the TIS. Apart from this, an increase around position -1000 bp relative to the TIS was detected. H3K18ac levels were not influenced by illumination (Offermann *et al.*, 2008). Indeed, the data of the present thesis corresponded to these observations. The increase at the distal promoter around position -1000 bp for example was observed in the present thesis, too. The fact that H3K18ac is not induced by illumination was observed in the present thesis as well.

In the present thesis, the H3K18ac distribution in maize demonstrated a similar course over the observed part of the gene in illuminated and darkened plants. Both showed increased acetylation levels at position -1050 bp relative to the TIS, at the distal promoter and at position -200 bp, at the proximal promoter. The acetylation level of H3K18 decreased again between position -200 bp and the beginning of the transcribed region, where lowest levels were measured at position +10 bp (Figure 3.1 D, left). Concerning R1001, H3K18ac showed a similar but more even distribution than in maize. In case of the distribution of H3K18ac in darkened plants of R1001, acetylation levels were increased at the distal promoter and the proximal promoter, but decreased between the proximal promoter and the beginning of the transcribed region. Although the distribution was more even than in maize, it showed strong similarities between R1001 and maize. Darkened plants of R1002 on the other hand, differed from maize. Here, the acetylation level was not increased at the distal promoter and the proximal promoter. Illuminated plants of R1002 also demonstrated an even distribution of H3K18ac without any detectable peaks (Figure 3.1 D, left).

In contrast to the previously described acetylations H3K9ac and H4K5ac which are known for their light inducibility, H3K18ac represents an example for an acetylation which is not induced by the influence of light. It was, therefore, included as example for an acetylation which is characterized by a different behavior than H3K9ac and H4K5ac. In fact, it was not possible to observe light induction for H3K18ac for maize in the present thesis, as well. This was also observed for R1001 and R1002, which even demonstrated an anti-correlation between illumination and acetylation levels.

To sum up, it was observed, that H3K18ac reacted to illumination in a similar way in both species. H3K18ac showed a broad distribution over the observed part of the gene and no light induction. In rice, it was even possible to observe a slight anti-correlation of H3K18ac to illumination. A correlation between mRNA levels in illuminated and darkened maize and rice to the acetylation of H3K18ac could, therefore, not be observed.

3.1.6 The H3K4me3 level of C4-*PEPC* in maize and transgenic rice under the influence of illumination or darkness

In contrast to the acetylation of the histone lysine residues H3K9 and H4K5, the methylation state of histones is independent of light induction and transcriptional activity (Danker *et al.*, 2008) Concerning the distribution of H3K4me3, previous data demonstrated that H3K4me3 can be found at the proximal promoter and at the transcribed region, where it occurs independent of the genes activation status. The intergenic regions and the distal promoter on the contrary are characterized by absence of H3K4me3 (Danker *et al.*, 2008).

Concerning H3K4me3, similarities between the two species were the absence of the trimethylation at the distal promoter and an increase of methylation levels between the proximal promoter and the beginning of the transcribed region. This corresponded to previously known data, as described above. Concerning the distribution of H3K4me3 over the observed part of C4-*PEPC* in maize, it was observed that signals were only weak at the distal promoter. Beginning at position -400 bp, the levels of H3K4me3 were constantly increasing to reach a maximum at position +10 bp at the beginning of the transcribed region. A 13-fold increase was observed for H3K4me3 levels between positions -400 bp and +10 bp relative to the TIS in illuminated plants. Darkened plants of maize showed the same course as illuminated plants. Again, H3K4me3 was absent at the distal promoter and increased towards the beginning of the transcribed region. A 30-fold increase was observed for H3K4me3 between said positions in darkened maize (Figure 3.1 F, left). The distribution of H3K4me3 over the observed part of C4-*PEPC* showed similarities between the control species maize and transgenic rice. As in maize, H3K4me3 showed only low signals at the distal promoter in illuminated R1001 and R1002. Already beginning at position -900 bp (which is further upstream than in the case of maize), H3K4me3 levels began to increase in both R1001 and R1002. There was a constant increase of H3K4me3 towards the beginning of the transcribed region. In R1001, a 9-fold increase of H3K4me3 was measured between positions -900 bp and +10 bp in illuminated plants. In darkened R1001 H3K4me3 levels demonstrated the same course as in illuminated plants with a 16-fold increase between positions -900 bp and +10 bp. For R1002, observations were similar to the results of R1001. H3K4me3 levels in illuminated R1002 showed a 12-fold increase between position -500 bp towards position +10 bp in illuminated plants and a 6-fold increase between positions -500 bp and +10 bp in darkened plants (Figure 3.1 F, left).

Concerning light inducibility, it was observed that in maize, H3K4me3 levels were slightly higher in darkened plants, between positions -500 bp and +10 bp. According to previous results, light

induction could, therefore, not be observed. Like maize, transgenic rice did not show light induction of the trimethylation H3K4me3.

To sum up, the distribution of H3K4me3 over the observed part of C4-*PEPC* was similar in maize and rice. No light induction was observed for H3K4me3 levels in maize and rice, corresponding to the expectations based on previous studies. Instead, maize showed a slight reduction of H3K4me3 in illuminated rice plants, beginning at position -500 bp. A correlation between the mRNA level and the trimethylation H3K4me3 could, therefore, not be observed.

3.2 The influence of illumination or darkness on the mRNA level and chromatin state of C4-*PPDK* in maize and transgenic rice

In addition to C4-*PEPC*, another important gene of the C4-mechanism has been evaluated with regard to its reaction to the illumination status in transgenic rice. The influence of illumination on the transcriptional activity and the chromatin state of C4-*PPDK* has already been described in detail: It is known that illumination is one of various stimuli which regulate C4-*PPDK* expression in maize plants. The influence of light strongly induces the transcriptional activity of C4-*PPDK* (Sheen, 1999; Horst, 2009; Heimann *et al.*, 2013). Therefore, the light inducibility of C4-*PPDK* was also part of the present thesis, as it provides a further aspect to be compared between the C4-plant maize and transgenic rice, expressing C4-genes. Here, the focus was set on the ability of the transgenic rice lines R1007 and R1008, which are expressing maize C4-*PPDK*, to regulate the gene in the same way, in which it would be regulated in the donor species maize. To investigate the regulation of C4-*PPDK* in transgenic rice with regard to the illumination status, R1007 and R1008 were harvested after 1 h of illumination and after 48 h of prolonged darkness. Maize was used as a control. It was treated and harvested according to the procedure for transgenic rice. The plant material was either used for RNA isolation (2.2.6), followed by subsequent cDNA-Synthesis (2.2.7), to evaluate the mRNA level of C4-*PPDK*, or it was used for crosslinking experiments (2.2.3) with subsequent ChIP (2.2.5) to evaluate the chromatin status with regard to the influence of illumination or prolonged darkness. To compare the chromatin state of C4-*PPDK* in maize and transgenic rice, a profile of the nucleosome density on the gene locus has been recorded. The nucleosome density was calculated as percent (%) of input (Figure 3.2 B). The histone modifications H3K9ac (Figure 3.2 C), H4K5ac (Figure 3.2 E) and H3K4me3 (Figure 3.2 D) were evaluated with regard to the nucleosome density. The profile ranged from the distal promoter at position -1200 bp on the upstream promoter, to position +85 bp in the transcribed region of C4-*PPDK*. For a more detailed comparison, two representative positions (for the distal promoter at position -760 bp, for the proximal promoter at position -150 bp) are shown for the control plant *Zea mays* as well as for R1007 and R1008. These positions have been chosen as they were evaluated for C4-*PPDK* in detail in previous studies (Heimann *et al.*, 2013) and, therefore, provided a suitable basis for comparisons to previously known information on C4-*PPDK*. The results of the reaction of C4-*PPDK* to illumination are presented in Figure 3.2.

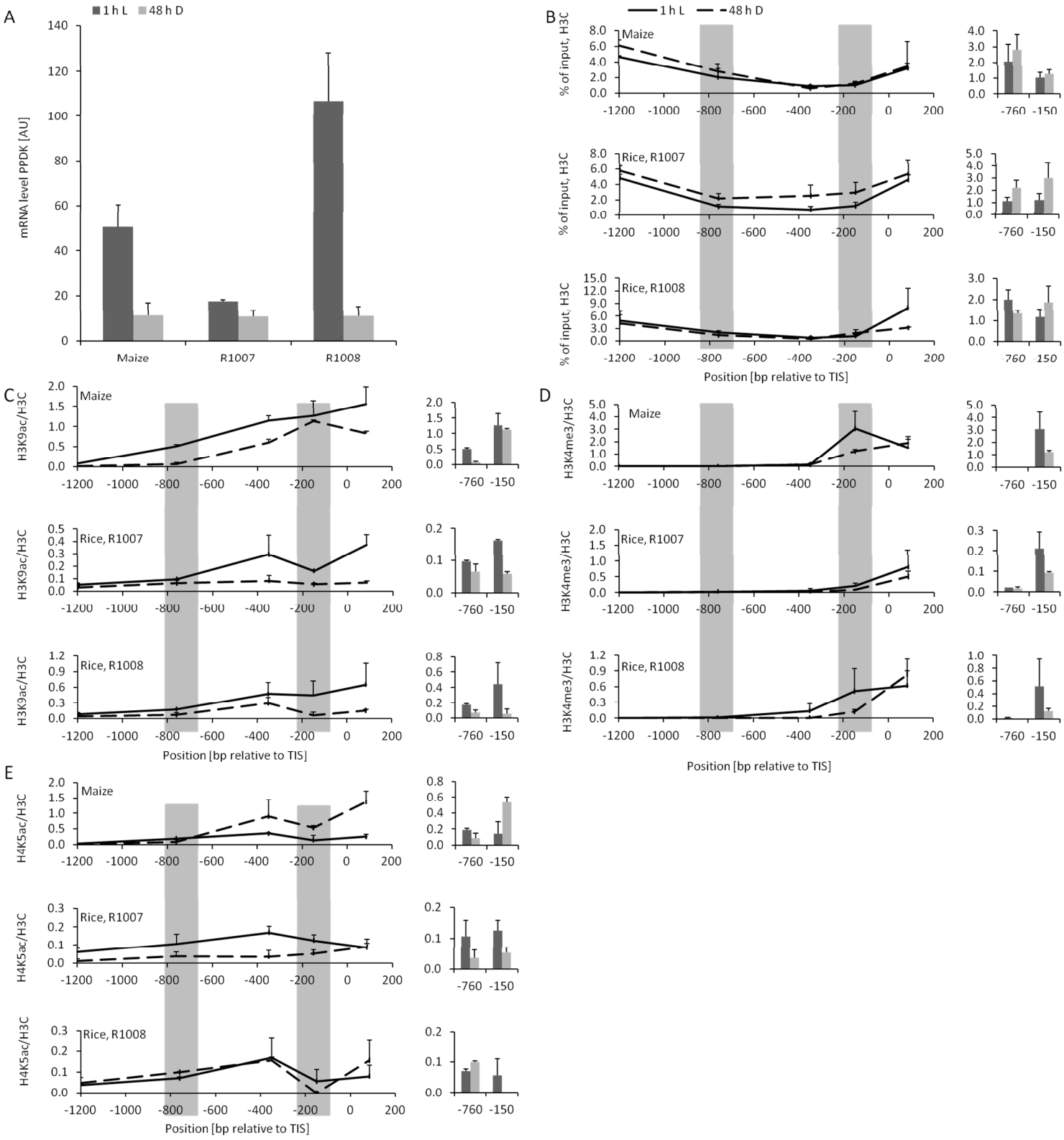
A
1 h L
48 h D
mRNA level PPDK [AU]
Maize
R1007
R1008
B
1 h L
48 h D
% of input, H3C
Maize
Rice, R1007
Rice, R1008
Position [bp relative to TIS]
C
H3K9ac/H3C
Maize
Rice, R1007
Rice, R1008
Position [bp relative to TIS]
D
H3K4me3/H3C
Maize
Rice, R1007
Rice, R1008
Position [bp relative to TIS]
E
H4K5ac/H3C
Maize
Rice, R1007
Rice, R1008
Position [bp relative to TIS]

Figure 3.2: mRNA and histone modification levels of C4-*PPDK* in maize and transgenic rice under the influence of illumination. Chromatin was precipitated with antibodies directed against the acetylations at histone 3, lysine 9 (H3K9ac) and histone 4, lysine 5 (H4K5ac), and the trimethylation at histone 3 lysine 4 (H3K4me3). To evaluate the nucleosome density, an antibody against the invariant C-terminal end of histone 3 (H3C) has been used. For the nucleosome density, the amount is presented as percent (%) of input. The modifications were standardized on the nucleosome density. The results refer to plants harvested after 1 h of illumination (depicted as "1 h L") and plants that were kept in darkness for 48 h before they were used for crosslinking and subsequent ChIP (depicted as "48 h D"). (A) mRNA levels of C4-*PPDK* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1007 and R1008. (B) H3C levels of C4-*PPDK* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1007 and R1008. (C) H3K9ac levels of C4-*PPDK* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1007 and R1008. (D) H3K4me3 levels of C4-*PPDK* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1007 and R1008. (E) H4K5ac levels of C4-*PPDK* in illuminated and darkened maize and the illuminated and darkened transgenic rice lines R1007 and R1008. Data points are based on two independent experiments for ChIP experiments and on at least two independent experiments for mRNA levels. Vertical lines indicate standard errors.

3.2.1 The mRNA level of C4-*PPDK* in maize and transgenic rice under the influence of illumination or darkness

Corresponding to what was already known for the regulation of the transcriptional activity of C4-*PPDK* it was possible to observe a strong light induction of the mRNA level in maize (Figure 3.2 A, left). This effect could also be observed in R1007 and R1008 (Figure 3.2 A, right). The effect was strongest in R1008, in which a decrease by 90 % was observed, when the plants were exposed to 48 h of prolonged darkness. In R1007, the reduction upon darkness was lower, by only 38 %. In the donor species maize, a reduction of the mRNA level by 77 % was observed when the plants were kept in darkness for 48 h.

3.2.2 The nucleosome density of C4-*PPDK* in maize and transgenic rice under the influence of illumination or darkness

The distribution of the nucleosome density over the observed part of C4-*PPDK* was similar in the control plant maize and transgenic rice, demonstrating increased levels at the distal promoter and the transcribed region. It was, therefore, similar to the nucleosome density in the case of C4-*PEPC,* as described above. Concerning the reaction towards the illumination status in maize, it was not possible to detect differences in the nucleosome density between illuminated and darkened plants. In the case of transgenic rice, darkened and illuminated plants showed a similar nucleosome density (Figure 3.2 B).

3.2.3 The H3K9ac level of C4-*PPDK* in maize and transgenic rice under the influence of illumination or darkness

The acetylation status of H3K9 on the C4-gene *PPDK* was evaluated in maize and the transgenic rice lines R1007 and R1008 (Figure 3.2 C). In previous studies, H3K9ac was characterized by an increase between the upstream promoter and the beginning of the transcribed region of C4-*PPDK*. While the acetylation level of H3K9 that was measured at the proximal promoter demonstrated high values at positions -150 bp and -800 bp relative to the TIS, levels declined further upstream (Heimann *et al.*, 2013). Concerning the reaction of the acetylation level at said positions to the illumination status, it was observed that H3K9ac showed a similar regulation at both promoter positions. Both, the distal promoter and the proximal promoter demonstrated decreasing H3K9ac levels in darkened plants (Horst, 2009; Heimann *et al.*, 2013), or light induction of the acetylation at said positions, respectively.

Concerning the distribution of H3K9ac on C4-*PPDK* in maize, the results of the present thesis corresponded to previous studies. The acetylation level increased between the distal promoter and the proximal promoter and showed lowest acetylation levels at position -1200 bp relative to the TIS. This increase was constant and resulted in maximal values at position +85 bp relative to the TIS, in the transcribed region (Figure 3.2 C, left). A similar distribution was documented by Heimann *et al*. (2013). It was observed that the distribution was similar in both R1007 and R1008. In general, transgenic rice demonstrated an increase in acetylation levels of H3K9 between the distal promoter and the transcribed region. For R1007, this increase was not as even and constant as in maize and R1008, as levels decreased again at position -150 bp. In illuminated plants, there was altogether a 20-fold increase in maize, and a 7 to 8-fold increase in transgenic rice between positions -1200 and +85. In darkened maize, the acetylation level began to increase at position -760 bp and reached a 13-fold increased level at position +85 bp relative to the TIS. In darkened rice, there was a 2 to 4-fold increase between positions -1200 bp and +85 bp relative to the TIS (Figure 3.2 C).

In both species, nearly all positions demonstrated light induction for H3K9ac, except for position -150 bp in maize. In rice, this induction was less pronounced at the distal promoter, further upstream than position -760 bp (Figure 3.2 C). The distal promoter (-760 bp) showed an 8-fold induction of acetylation levels in illuminated plants for maize. In rice, the light induction of H3K9ac at the distal promoter was weaker than in maize (2-fold for both R1007 and R1008) while the light induction of H3K9ac at the proximal promoter, at position -150 bp relative to the TIS, was stronger than in maize. In R1007, acetylation levels at position -150 bp demonstrated a 3-fold induction upon illumination.

For R1008, this effect was even stronger. Here, H3K9ac levels at position -150 bp relative to the TIS, showed an 8-fold induction upon illumination.

To sum up, it could be concluded, that the results of the control species maize corresponded to what was already known from previous studies (Horst, 2009; Heimann *et al.*, 2013). Concerning the distribution of the histone modification over the observed part of the gene, as well as the reaction towards illumination, similarities were observed between maize and transgenic rice. Both species demonstrated a correlation between mRNA levels and acetylation levels of H3K9 in response to illumination and prolonged darkness. In rice, this correlation was pronounced at the proximal promoter and at the beginning of the transcribed region and weaker at the distal promoter around position -760 bp relative to the TIS.

3.2.4 The H4K5ac level of C4-*PPDK* in maize and transgenic rice under the influence of illumination or darkness

The acetylation status of H4K5ac was evaluated for C4-*PPDK* in maize and the transgenic rice lines R1007 and R1008. In previous studies, H4K5ac demonstrated decreasing H4K5ac levels in plants that were kept in prolonged darkness. This decrease was observed at both promoter positions, the distal and the proximal promoter, although it was less pronounced at the proximal promoter (Horst, 2009; Heimann *et al.*, 2013).

In the present thesis, the distribution of H4K5ac over the observed part of C4-*PPDK* in maize differed from what was documented by Heimann *et al.* (2013). Instead of increasing between the distal promoter and the transcribed region, H4K5ac levels stayed constant in illuminated maize plants and did not show regions with increased levels of H4K5ac. In darkened maize on the contrary, it was possible to observe a general increase of H4K5ac levels from the distal promoter towards the beginning of the transcribed region. In this context, the expected distribution was observed (Figure 3.2 E, left). R1007 demonstrated a slight increase in acetylation levels for H4K5 between the distal promoter and the proximal promoter. It reached a maximum at position -300 bp relative to the TIS. Then the acetylation level decreased again between the proximal promoter and the beginning of the transcribed region. The distribution of H4K5ac in darkened specimens of R1007 was distributed more evenly and in general showed a lower level than the acetylation in illuminated plants. A similar distribution was observed for R1008. Both illuminated and darkened R1008 showed increasing H4K5ac levels between the distal promoter and position -300 bp relative to the TIS. The level dropped again towards position -150 bp relative to the TIS (Figure 3.2 E, left). When the acetylation

levels of H4K5 at the two representative positions -760 bp relative to the TIS at the distal promoter and -150 bp relative to the TIS at the proximal promoter were evaluated, the distal promoter in maize corresponded to previous results, as it demonstrated light induction. The proximal promoter on the other hand differed from the expectations, as it showed decreased acetylation levels for H4K5ac at position -150 bp, while Heimann *et al.* (2013) also documented light inducibility of H4K5ac at this position. The results of R1007 on the other hand corresponded to previous results of H4K5ac in maize. Both positions demonstrated light induction for H4K5ac. For the proximal promoter, this effect could also be observed in R1008. Here, H4K5ac was reduced at position -150 bp in darkened plants. At position -760 bp the level of H4K5ac was similar in illuminated and darkened plants and showed slightly increased acetylation levels in darkened plants (Figure 3.2 E, right).

Summarizing, the distribution of H4K5ac was similar in maize and transgenic rice. Concerning the reaction towards illumination, it has to be noted that H4K5ac in maize did not completely react as expected based on the results of previous studies. Nevertheless, H4K5ac in R1007 demonstrated results that underlined the light inducibility at the distal and proximal promoter, as it was reported in previous studies (Heimann *et al.*, 2013). Therefore, R1007 showed a correlation between the mRNA level, which was increased under illumination, and the acetylation level at the proximal promoter. For the proximal promoter at position -150 bp relative to the TIS, this could also be observed for R1008. These results correspond to the previously described behavior of H4K5ac in response to illumination and darkness in the control species maize. Concerning the distal promoter, a correlation between the acetylation level and the mRNA level could only be observed for R1007. R1008 on the contrary showed no correlation for the acetylation level at this position and the mRNA level.

3.2.5 The H3K4me3 level of C4-*PPDK* in maize and transgenic rice under the influence of illumination or darkness

Apart from the two acetylations H3K9ac and H4K5ac, the well-characterized histone modification H3K4me3 was also included in the present thesis. In previous studies, H3K4me3 on C4-*PPDK* could not be detected at position -800 bp of the distal promoter (Heimann *et al.*, 2013). The H3K4me3 level is in general known to be independent of illumination (Danker *et al.*, 2008).

The distribution of H3K4me3 was very similar in maize and transgenic rice. It also corresponded to what was previously known for the typical distribution of H3K4me3 over gene bodies. In both species, the trimethylation level increased between the upstream promoter and the beginning of the transcribed region (Figure 3.2 D, left). Light induction could neither be observed for maize, nor for

transgenic rice (Figure 3.2 D, right). Therefore, there was no correlation between the trimethylation level at H3K4 and the mRNA level, independent of the species.

3.3 The diurnal rhythm of the mRNA level and chromatin state of C4-*PEPC* in maize and transgenic rice

In addition to the influence of external stimuli like illumination or the availability of nutrients like nitrogen, the transcriptional activity as well as the chromatin state of C4-genes are controlled by internal signals, too (Thomas *et al.*, 1990; Danker *et al.*, 2008; Horst *et al.*, 2009; Heimann *et al.*, 2013). One of these internal signals is the presence of an internal diurnal rhythm, which does not only establish changing mRNA and hnRNA levels of C4-*PEPC* throughout the day and the night (Horst *et al.*, 2009; Heimann *et al.*, 2013), but also shows an influence on the level of histone modifications (Horst *et al.*, 2009).

In order to find out whether C4-*PEPC* gene regulation in transgenic rice corresponds to its regulation in the donor species maize, it was tested if the mRNA level and the chromatin state of C4-*PEPC* in R1001 demonstrate a diurnal regulation which is similar to the diurnal rhythm in maize. For this purpose, 2-week old plants were harvested and crosslinked after 4 h of illumination and after 16 h of illumination. As further comparison, plants that were darkened for 48 h were included into the experiments. As a control, the experiments were also performed with 2-week old maize plants. As the results of the light induction of C4-*PEPC* and C4-*PPDK* revealed in general similar effects for the mRNA level of different transgenic rice lines, the experiments that were concentrating on the evaluation of a diurnal rhythm of C4-*PEPC* in transgenic rice were only performed with R1001. As the observed differences for the histone modifications evaluated for R1002 did not lead to a different reaction of the mRNA level, R1002 was not included. The plant material was either used for RNA extraction (2.2.6), followed by subsequent cDNA-Synthesis (2.2.7), to evaluate the mRNA level of C4-*PEPC*, or it was used for crosslinking (2.2.3) experiments with subsequent ChIP (2.2.5) to evaluate the chromatin status at two different time points throughout the day and in darkened plants. To compare the chromatin state of C4-*PEPC* in maize and transgenic rice, the nucleosome density on the gene locus has been recorded for three representative positions at the distal promoter (-1050 bp relative to the TIS), the proximal promoter (-200 bp relative to the TIS), and at the beginning of the transcribed region (+10 bp relative to the TIS) (Figure 3.3 B). The nucleosome density was calculated as % of input. The histone modifications H3K9ac (Figure 3.3 C), H4K5ac (Figure 3.3 E), H3K18ac (Figure 3.3 D) and H3K4me3 (Figure 3.3 F) were evaluated with regard to the nucleosome density. The representative positions at -1050 bp for the distal promoter, -200 bp for the proximal promoter and +10 bp for the beginning of the transcribed region are shown for both the control species *Zea mays* and R1001. The results of the control species maize in general corresponded to the findings described by Horst (2009) for the histone acetylations and, as far as darkened and 4 h illuminated

plants were concerned, also to the results of the present thesis (0). Therefore, the data for 4 h illuminated plants and darkened plants are not described in detail again, but are used as controls in the present experiment. In this part of the results, the focus is set on the comparison of 16 h illuminated plants to 4 h illuminated plants and plants that have been darkened for 48 h. This approach is used to evaluate the diurnal regulation of C4-*PEPC* in transgenic rice, for which a comparison to a situation with increased transcriptional activity (4 h of illumination) and to a situation with hardly any transcriptional activity or no transcriptional activity (48 h of darkness) is useful.

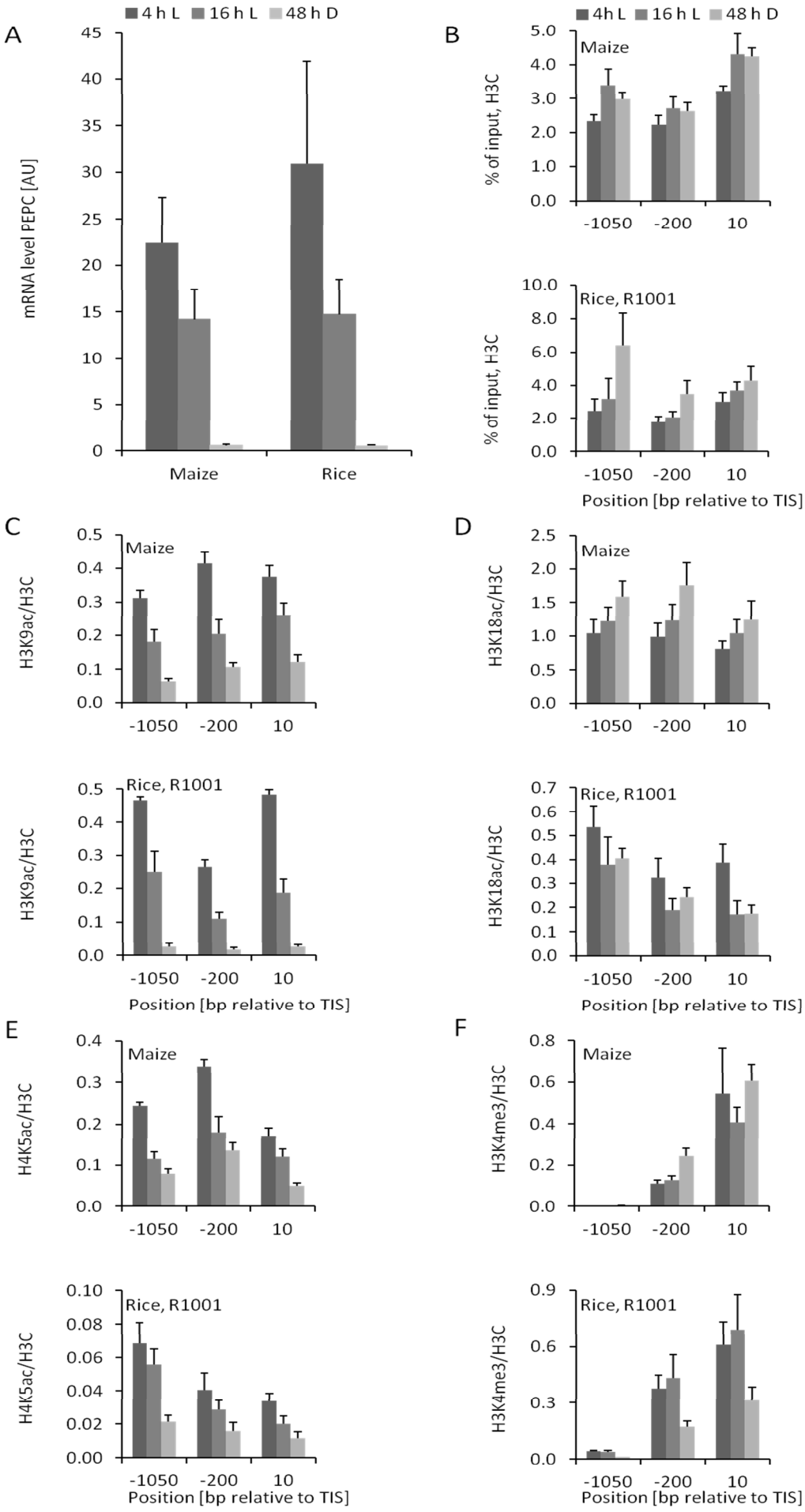
A
4 h L
16 h L
48 h D
mRNA level PEPC [AU]
Maize
Rice
B
% of input, H3C
Maize
Rice, R1001
-1050
-200
10
Position [bp relative to TIS]
C
H3K9ac/H3C
Maize
Rice, R1001
D
H3K18ac/H3C
E
H4K5ac/H3C
F
H3K4me3/H3C

Figure 3.3: mRNA and histone modification levels of C4-*PEPC* in maize and transgenic rice at different time points. Chromatin was precipitated with antibodies directed against the acetylations at histone 3, lysine 9 (H3K9ac) and lysine 18 (H3K18ac), histone 4, lysine 5 (H4K5ac), and the trimethylation at histone 3 lysine 4 (H3K4me3). To evaluate the nucleosome density, an antibody against the invariant C-terminal end of histone 3 (H3C) has been used. For the nucleosome density, the amount is presented as percent (%) of input. The modifications were standardized on the nucleosome density. The results refer to plants harvested after 4 h of illumination (depicted as "4 h L"), plants harvested after 16 h of illumination (depicted as "16 h L") and plants that were kept in darkness for 48 h before they were used for crosslinking and subsequent ChIP (depicted as "48 h D"). (A) mRNA levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. (B) H3C levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. (C) H3K9ac levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. (D) H3K18ac levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. (E) H4K5ac levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. (F) H3K4me3 levels of C4-*PEPC* in 4 h and 16 h illuminated and 48 h darkened maize and the 4 h and 16 h illuminated and 48 h darkened transgenic rice line R1001. Data points are based on at least three independent experiments for ChIP experiments and on at least three independent experiments for mRNA levels. Vertical lines indicate standard errors.

3.3.1 Diurnal regulation of C4-*PEPC* mRNA in maize and transgenic rice

A comparison of the mRNA level in the donor species maize and the transgenic rice line R1001 revealed that in both species the C4-*PEPC* mRNA level sank between 4 h of illumination and 16 h of illumination. In maize, the mRNA level measured after 16 h of illumination accounted for 63 % of the mRNA level measured after 4 h of illumination (Figure 3.3 A, left). This result corresponded to the findings of Horst (2009). In R1001, the mRNA level decreased to 48 % after 16 h of illumination (Figure 3.3 A, right). Therefore, it was observed that the reduction in C4-*PEPC* mRNA throughout the day is even stronger than in the control species maize. As expected, darkened specimens of both species demonstrated more than 95 % reduction of C4-*PEPC* mRNA levels, each. This corresponded to the results presented in 3.1.1, in which darkened plants demonstrated a similar reduction of mRNA levels.

It can, therefore, be concluded that the reduction of C4-*PEPC* mRNA levels in the course of a diurnal rhythm is similar in maize and transgenic rice.

3.3.1 Diurnal regulation of the nucleosome density of C4-*PEPC* in maize and transgenic rice

To compare the chromatin state in maize and transgenic rice, the nucleosome density has been measured. The distribution of H3C in maize control plants and rice plants which have been illuminated for 4 h, or kept in prolonged darkness for 48 h, corresponded to the data already described during the evaluation of the light induction of C4-*PEPC* in transgenic rice (3.1.2). For maize,

it also corresponded to the results of intact control plants in Offermann *et al.* (2008). In said study, the nucleosome density was slightly increased at the distal promoter and at the beginning of the transcribed region, in comparison to the nucleosome density at the proximal promoter (Offermann *et al.*, 2008).

As the nucleosome density in darkened plants and plants that have been illuminated for 4 h has already been described in 3.1.2 for maize and rice, it is not described in detail at this point. As in the aforementioned experiments, the nucleosome density did not change upon illumination or prolonged darkness. In the present experiment, 16 h illuminated plants were included. For these plants, the nucleosome density was slightly higher than in 4 h illuminated plants (Figure 3.3 B).

With regard to R1001 it was observed that the nucleosome density of 4 h illuminated plants and darkened plants showed a similar distribution for the positions -1050 bp, -200 bp and +10 bp relative to the TIS, as the control plant maize did and as it was described in 3.1.2. Nevertheless, in the present experiment, the values for darkened plants were higher than in illuminated plants. For 4 h and 16 h illuminated transgenic rice plants, there was no difference in the nucleosome density.

Summarizing, the nucleosome density on C4-*PEPC* was independent of a diurnal rhythm in both species and showed a similar distribution with regard to the three representative positions of the gene in maize and transgenic rice, independent of the time point at which it was harvested and crosslinked. Nevertheless, both species demonstrated reduced mRNA levels after 16 h of illumination and if the plants were kept in prolonged darkness.

3.3.2 Diurnal regulation of H3K9ac of C4-*PEPC* in maize and transgenic rice

In addition to the mRNA level of C4-*PEPC*, the acetylation level of H3K9 has been evaluated for plants that have been illuminated for 4 h and for plants that have been illuminated for 16 h. Again, darkened plants were included for comparison and the experiment was performed with maize as control (Figure 3.3 C).

The results of maize corresponded to the observations of Horst *et al.* (2009). A reduction of H3K9ac throughout the illumination period was observed for all of the tested positions. Lowest acetylation levels were observed for darkened control plants. Although the acetylation levels in maize after 16 h of illumination were not as low as in plants kept in prolonged darkness, there was a clear reduction of H3K9ac. In maize, this reduction was strongest at the proximal promoter, at position -200 bp relative to the TIS. Here, H3K9ac levels after 16 h of illumination accounted for 49 % of the initially

measured H3K9ac level after 4 h of illumination. At the distal promoter and at the beginning of the transcribed region, the reduction of acetylation levels was weaker than at the proximal promoter. At position -1050 bp relative to the TIS, H3K9ac levels after 16 h of illumination were 58 % of the H3K9ac levels after 4 h of illumination, while they accounted for 70 % of the H3K9ac levels after 4 h of illumination at the beginning of the transcribed region.

A reduction of H3K9ac levels after 16 h of illumination could also be observed in R1001. This reduction was measured at all of the tested positions and was stronger than in the control species maize. The strongest reduction was observed at the beginning of the transcribed region, were acetylation levels after 16 h of illumination reached 39 % of the levels measured after 4 h of illumination. At the proximal promoter and the distal promoter, H3K9ac levels reached 41 % (-200 bp), or 54 % (-1050 bp) of the H3K9ac levels after 4 h of illumination. As expected, lowest acetylation levels were measured in darkened plants.

Summarizing, the results of H3K9ac corresponded to what was first observed by Horst (2009) and were similar in both species. They demonstrated a correlation to mRNA levels at all of the tested positions.

3.3.3 Diurnal regulation of H4K5ac of C4-*PEPC* in maize and transgenic rice

As in the case of H3K9ac, previous studies demonstrated a diurnal regulation for H4K5ac in maize which was characterized by a decrease in acetylation levels after 16 h of illumination at all of the tested positions, including the complete promoter region as well as positions within the transcribed region (Horst *et al.*, 2009). The results of the present thesis in general corresponded to the data already known for H4K5ac in maize.

As the results of H4K5ac in 4 h illuminated plants and for darkened plants corresponded to previously described data (3.1.4), they are not explained in detail at this point. Concerning a diurnal regulation and reduction of H4K5ac, which is independent of the illumination status, it was observed that the present results corresponded to the data described by Horst *et al.* (2009). All of the tested positions demonstrated a clear decrease in acetylation levels at H4K5. In the case of maize, this reduction of acetylation levels was nearly as strong as the reduction of the modification in darkened plants. The strongest reduction was measured at the distal promoter. After 16 h of illumination, H4K5ac levels at this position accounted for 47 % of the level in plants that have only been illuminated for 4 h. At the proximal promoter, the H4K5ac level was reduced to 53 %, while at the beginning of the transcribed region, the level was reduced to 70 % (Figure 3.3 E).

R1001 also demonstrated a diurnal regulation and thus reduction of H4K5ac at all of the tested positions. The diurnal regulation of this modification was, therefore, similar in transgenic rice and in the control species maize. In rice, the reduction was not as strong as in maize. H4K5ac levels were reduced to 81 % at the distal promoter, to 71 % at the proximal promoter and to 60 % at the beginning of the transcribed region.

It can, therefore, be concluded that both species showed a diurnal regulation of H4K5ac, which was characterized by a reduction after 16 h of illumination. The acetylation, therefore, correlated with the mRNA level in both species.

3.3.4 Diurnal regulation of H3K18ac of C4-*PEPC* in maize and transgenic rice

Additionally, the histone acetylation H3K18ac, which is known to be independent of illumination (Offermann *et al.*, 2008), has been included in the evaluation of the diurnal C4-*PEPC* rhythm in transgenic rice.

Previous data by Horst (2009) demonstrated that the modification H3K18ac showed an anti-correlation to illumination and similar acetylation levels for C4-*PEPC* in 4h and 16 h illuminated plants, except for the distal promoter at which the difference between 4 h and 16 h illuminated plants was higher. The results of the present thesis in general correspond to the previously described data (Horst *et al.*, 2009), except for position -1050, for which 4 h illuminated plants and 16 h illuminated plants were more similar in the present thesis. Apart from this, the results of 4 h illuminated plants and darkened plants corresponded to the observations which have been described in 3.1.5 in the context of the evaluation of C4-*PEPC* light induction in transgenic rice. They are, therefore, not described in detail at this point.

Concerning the presence of a diurnal rhythm of H3K18 acetylation, it was observed that the control plant maize did not show any differences between 4 h and 16 h illuminated plants. While Horst (2009) described a reduction in H3K18ac levels in the distal promoter, beginning at position -1000 bp relative to the TIS and further upstream, it was not possible to detect this decrease in the present thesis. Due to the limitations caused by the C4-*PEPC* construct introduced into rice, it was not possible to investigate if said reduction would be detectable further upstream than -1050 bp relative to the TIS. Concerning the proximal promoter and the beginning of the transcribed region on the other hand, the results of the control species maize corresponded to the expectations and a diurnal regulation could not be observed for H3K18ac.

With regard to the situation in transgenic rice, it was possible to observe similarities to the acetylation of H3K18 in maize. Notably, the anti-correlation with regard to illumination could not be observed. Apart from the beginning of the transcribed region, there was no reduction in H3K18ac levels after 16 h of illumination.

Summarizing, the results revealed no correlation between the mRNA level of C4-*PEPC*, which showed a diurnal regulation in both, maize and transgenic rice, and the acetylation level at H3K18ac for the distal promoter and the proximal promoter. Only the beginning of the transcribed region in transgenic rice demonstrated a reduction of H3K18ac levels after 16 h of illumination.

3.3.5 Diurnal regulation of H3K4me3 of C4-*PEPC* in maize and transgenic rice

Apart from the acetylation of H3K18, an additional modification which is also independent of illumination and transcriptional activity (Danker *et al.*, 2008) was included in the present thesis. The typical distribution of H3K4me3 over the gene body, as well as its reaction to illumination has already been described in 1.1.1. The results of 4 h illuminated plants and darkened plants of the control species maize corresponded to the described data and also mirrored the results of previous studies (Danker *et al.*, 2008; Offermann *et al.*, 2008). It was possible to detect the typical distribution of H3K4me3 in 16 h illuminated plants of maize and rice, which is characterized by absence at the distal promoter and increasing levels towards the proximal promoter and towards the beginning of the transcribed region (Figure 3.3 F). Both species demonstrated absence of the trimethylation at the distal promoter and increasing levels towards the beginning of the transcribed region. It was not possible to observe a diurnal rhythm of H3K4me3, which would be accompanied by a reduction of H3K4me3 throughout the day, in neither of the two species. In both maize and transgenic rice, the trimethylation levels of H3K4 were similar in plants that were illuminated for only 4 h and plants that were illuminated for 16 h. Therefore, the results demonstrated similarities for the two species and revealed no correlation of the H3K4me3 level to the mRNA level. Notably, the trimethylation level in darkened plants of R1001 was lower than the trimethylation level in 4 h and 16 h illuminated plants, which did not correspond to previous results of the present thesis (1.1.1) and to the results of previous studies (Offermann *et al.*, 2008).

Summarizing, H3K4me3 did not show reduced levels after 16 h of illumination in maize. This was also observed in the recipient species rice. Therefore, a correlation with the mRNA level throughout the time course could not be observed.

3.4 The influence of nitrogen depletion on the mRNA level and chromatin state of C4-*PEPC* in maize and transgenic rice

Apart from the influence of illumination on specific histone modifications, a series of further external and internal stimuli shows an impact on the chromatin state and the presence or absence of different histone modifications and on transcriptional activity. Offermann *et al.* (2008) investigated the influence of nitrogen depletion on the acetylation levels of several individual histone lysine residues, including H3K9ac, H4K5ac and H3K18ac. It is, therefore, known that the availability of nitrate for example influences the acetylation levels of H4K5, H3K9 and H3K18 among others. The transcription rate of C4-*PEPC*, which was measured via the amount of hnRNA, showed a strong decline, if plants were subjected to a treatment simulating nitrogen depletion. On the chromatin level, different reactions to the treatment were observed for the distal and the proximal promoter. While at the proximal promoter and at the beginning of the transcribed region, nitrogen depletion did not provoke changes in the acetylation level, it led to a 3 to 5-fold reduction of acetylation levels at the distal promoter. The transcriptional level and acetylation at the distal promoter are, therefore, co-regulated (Offermann *et al.*, 2008).

The present thesis aimed at gaining information about the behavior of C4-*PEPC* in transgenic rice. Therefore, transgenic rice was exposed to nitrogen depletion (2.2.2.3) and the effects on the transcriptional rate and the chromatin state were evaluated. As a control, the experiments were also performed with 3-week old maize plants. To investigate the regulation of C4-*PEPC* in transgenic rice with regard to the reaction towards nitrogen depletion, maize and rice plants were harvested after 4 h of illumination and after being cut and transferred to tap water after 1 h of illumination and additional 3 h of illumination while being incubated in tap water. Maize was used as a control. As the results of the light induction of C4-*PEPC* and C4-*PPDK* revealed in general similar effects for the mRNA level of different transgenic rice lines, the experiments from now on focused on R1001. As the observed differences to the histone modifications evaluated for R1002 did not lead to a different reaction of the mRNA level, R1002 was no longer included. The plant material was either used for RNA isolation (2.2.6), followed by subsequent cDNA-Synthesis (2.2.7), to evaluate the mRNA level of C4-*PEPC*, or it was used for crosslinking experiments with subsequent ChIP (2.2.5) to evaluate the chromatin status with regard to the influence of nitrogen depletion. To compare the chromatin state of C4-*PEPC* in maize and transgenic rice, a profile of the nucleosome density on the gene locus has been recorded (Figure 3.4 B). The nucleosome density was calculated as % of input. The histone modifications H3K9ac (Figure 3.4 C), H4K5ac (Figure 3.4 E), H3K18ac (Figure 3.4 D) and H3K4me3

(Figure 3.4 F) were evaluated with regard to the nucleosome density. Three representative positions at -1050 bp for the distal promoter, -200 bp for the proximal promoter and +10 bp for the beginning of the transcribed region are shown for the control plant *Zea mays* as well as for R1001. As the aim of the present thesis was to find out whether nitrogen depletion in general leads to a similar reaction of the transcriptional level and chromatin state in transgenic rice, crosslinking experiments have only been performed with intact control plants and plants suffering from nitrogen depletion. Specimens that have been treated with zeatin were, therefore, no longer included at this point. The results of the control experiment corresponded to the findings described by Offermann *et al.* (2008) for the histone modification H3K9ac. H4K5ac differed from what was previously known, as the distal promoter did not show a reduction of acetylation levels under nitrogen depletion. This could also be observed for H3K18ac in maize. Concerning the transcriptional rate, there was a decrease in both cases, in the present thesis and previous results, but to a stronger degree (80 %) in Offermann *et al.* (2008).

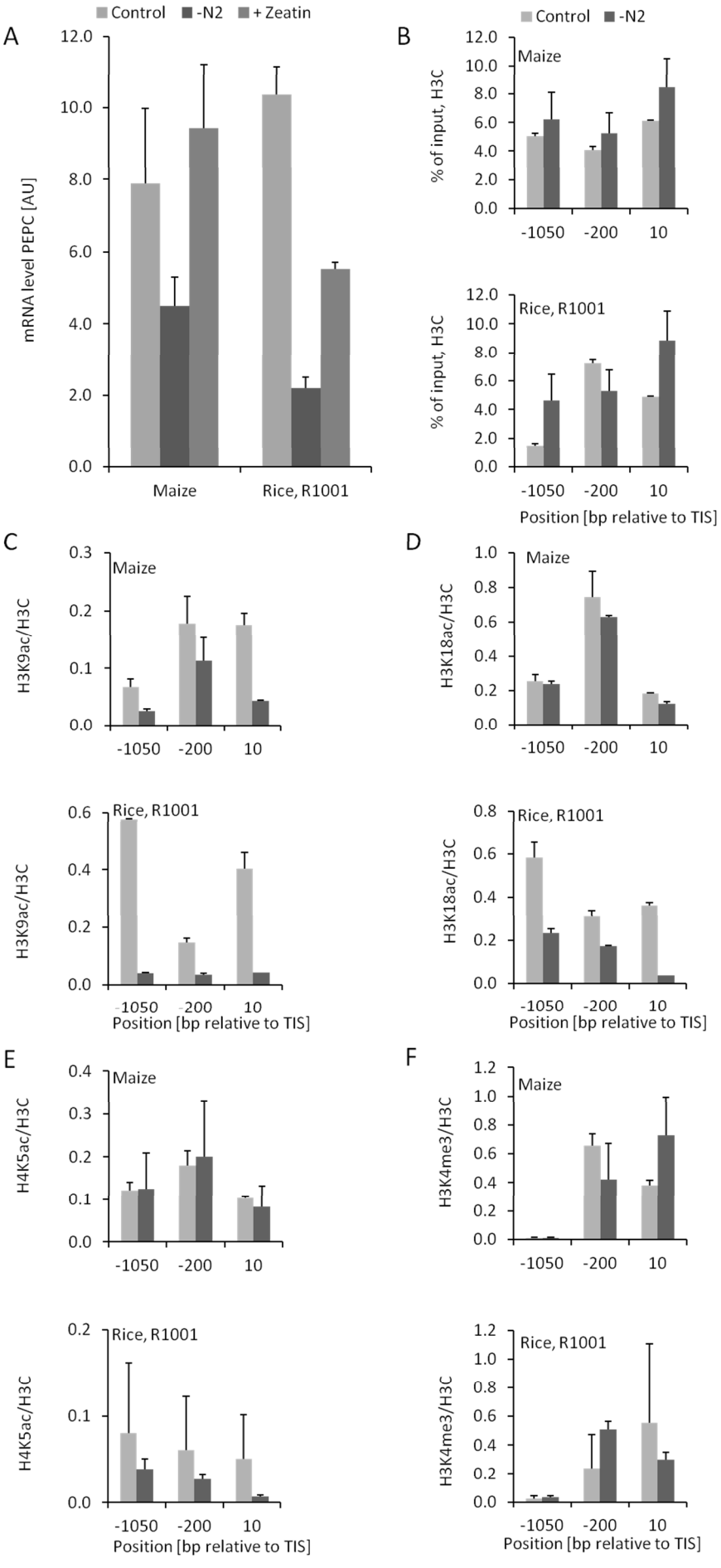
A
Control
-N2
+ Zeatin
mRNA level PEPC [AU]
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Maize
Rice, R1001
B
Control
-N2
Maize
% of input, H3C
Rice, R1001
-1050
-200
10
Position [bp relative to TIS]
C
H3K9ac/H3C
Maize
Rice, R1001
D
H3K18ac/H3C
Maize
Rice, R1001
E
H4K5ac/H3C
Maize
Rice, R1001
F
H3K4me3/H3C
Maize
Rice, R1001

Figure 3.4: mRNA and histone modification levels of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion. Chromatin was precipitated with antibodies directed against the acetylations at histone 3, lysines 9 and 18 (H3K9ac and H3K18ac), histone 4, lysine 5 (H4K5ac), and the trimethylation at histone 3 lysine 4 (H3K4me3). To evaluate the nucleosome density, an antibody against the invariant C-terminal end of histone 3 (H3C) has been used. For the nucleosome density, the amount is presented as percent (%) of input. The modifications were standardized on the nucleosome density. The results refer to plants harvested after 4 h of illumination (depicted as "Control") and plants that were transferred to tap water after 1 h of illumination and incubated in tap water for additional 3 h before they were used for crosslinking and subsequent ChIP (depicted as "–N2"). Plants that were incubated in tap water containing 0.5 µM of zeatin are indicated as "+Zeatin". (A) mRNA levels of C4-*PEPC* in intact, nitrogen depleted and zeatin-treated maize and in the intact, nitrogen depleted and zeatin-treated transgenic rice line R1001. (B) H3C levels of C4-*PEPC* in intact and nitrogen depleted maize and in the intact and nitrogen depleted transgenic rice line R1001. (C) H3K9ac levels of C4-*PEPC* in intact and nitrogen depleted maize and in the intact and nitrogen depleted transgenic rice line R1001. (D) H3K18ac levels of C4-*PEPC* in intact and nitrogen depleted maize and in the intact and nitrogen depleted transgenic rice line R1001. (E) H4K5ac levels of C4-*PEPC* in intact and nitrogen depleted maize and in the intact and nitrogen depleted transgenic rice line R1001. (F) H3K4me3 levels of C4-*PEPC* in intact and nitrogen depleted maize and in the intact and nitrogen depleted transgenic rice line R1001. Specimens that have been treated with zeatin were not included in the evaluation of histone modification levels as the comparison between maize and transgenic rice focused on the effects of nitrogen depletion. Data points are based on two independent experiments for ChIP experiments and maize mRNA and on three independent experiments for rice mRNA. They are presented for the representative positions -1050 bp, -200 bp and +10 bp relative to the TIS. Vertical lines indicate standard errors.

3.4.1 The mRNA level of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

When the mRNA level of C4-*PEPC* was compared between maize and rice, a decrease under nitrogen depletion was observed for both species (Figure 3.4 A). The results differed insofar, as there was a stronger decrease in rice, than in maize. In the control species maize, the incubation in tap water resulted in a 35 % decrease, while mRNA levels decreased by 75 % under the same conditions in rice. The effect was reversed in both species by the addition of 0.5 µM of trans-zeatin into the tap water (Figure 3.4 A). Trans-zeatin is a phytohormone which transports the information of available nitrogen from the roots to the tips of the plant (Sugiharto *et al.*, 1992; Dong *et al.*, 2016) and was, therefore, chosen as a positive control to underline that the observed reduction in plants kept in tap water was actually caused by nitrogen depletion. In this context, there were again differences between maize and rice. In the control plant maize, the added zeatin was not only able to fully restore the mRNA level, it also led to a further increase of the mRNA level. In transgenic rice, adding zeatin did not lead to a full recovery of the mRNA level. Nevertheless, 53 % of the mRNA level in intact plants was restored in zeatin-treated R1001 (Figure 3.4 A).

Summarizing it was observed that in both species, nitrogen depletion led to a reduction of the mRNA level. Adding zeatin led to increased mRNA levels in both species.

3.4.2 The nucleosome density of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

Offermann *et al.* (2008) observed an even distribution of H3C in intact control plants of maize and maize plants subjected to nitrogen depletion. The nucleosome density was slightly increased at the distal promoter and the beginning of the transcribed region, compared to the nucleosome density at the proximal promoter. This could also be observed in the present thesis. The level of the nucleosome density, which was measured as percent (%) of input, was similar in nitrogen depleted plants and control plants of maize. In rice, the distal promoter and the beginning of the transcribed region demonstrated an increased nucleosome density in response to nitrogen depletion (Figure 3.4 B).

The influence of nitrogen depletion on the nucleosome density was compared for the three representative positions -1050 bp, -200 bp and +10 bp relative to the TIS. For maize, the density was similar in nitrogen depleted plants and intact control plants as well for the distal promoter, as for the proximal promoter and the beginning of the transcribed region. (Figure 3.4 B). While in maize the nucleosome density was similar in nitrogen depleted plants and intact control plants at all three of the representative positions, this could not be observed for transgenic rice. Here the nucleosome density was higher in nitrogen depleted plants at positions -1050 bp and +10 bp relative to the TIS. It showed a 2-fold increase at position -1050 bp when the plants were suffering from nitrogen depletion. Concerning the beginning of the transcribed region, at position +10 bp, the nucleosome density showed a 2-fold increase under nitrogen depletion. A different development was observed for the proximal promoter at position -200 bp relative to the TIS. Here, intact control plants showed a similar nucleosome density to nitrogen depleted plants. At this position, it was in general possible to observe the highest nucleosome density for intact control plants of transgenic rice. It can be concluded that the difference in the reaction of C4-*PEPC* mRNA in transgenic rice, which showed a stronger response to nitrogen depletion that C4-*PEPC* in the donor plant maize, is accompanied by an increased nucleosome density at the distal promoter and the beginning of the transcribed region on C4-*PEPC* in transgenic rice.

3.4.3 The H3K9ac level of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

The acetylation level of H3K9 has been documented for nitrogen depleted plants and intact control plants, for maize and transgenic rice expressing C4-*PEPC* (Figure 3.4 C).

Offermann *et al.* (2008) described the impact of nitrogen depletion on a series of different lysine residues of H3 and H4 in maize, for example including H4K5ac, H3K9ac and H3K18ac. In the context of this study, it could be revealed that the distal promoter and the proximal promoter react in a different way to nitrogen depletion. All of the lysine residues that were included in the experiments demonstrated a 3 to 5-fold reduction of acetylation levels on the distal promoter, if the plants were suffering from nitrogen depletion. The proximal promoter on the other hand showed constant levels of acetylation, independent of the nitrogen availability (Offermann *et al.*, 2008). With regard to the donor species maize, the results of the present thesis corresponded to the findings described by Offermann *et al.* (2008) for the histone modification H3K9ac. H4K5ac differed from what was previously known, as the distal promoter did not show a reduction of acetylation levels under nitrogen depletion. This could also be observed for H3K18ac in maize.

On the basis of the results provided by (Offermann *et al.*, 2008) it was expected that the acetylation level of H3K9 would react to nitrogen depletion at the distal promoter with a reduction, while the acetylation level of H3K9 at the proximal promoter should be uninfluenced by nitrogen depletion (Offermann *et al.*, 2008). For the distal promoter, at position -1050 bp, this effect was also observed in maize plants of the present thesis. At position -1050 bp relative to the TIS, nitrogen depletion led to a 63 % decrease in maize. This result corresponded to previous data provided by Offermann *et al.* (2008). According to previous results, the acetylation level at position -200 bp relative to the TIS should be uninfluenced by nitrogen depletion. This could also be observed in the present thesis, as the acetylation level stayed constant at the proximal promoter. For the beginning of the transcribed region on the other hand, it was possible to observe a reduction in acetylation levels under the influence of nitrogen depletion.

Concerning R1001, the acetylation level of H3K9ac was clearly reacting to nitrogen depletion at all three of the tested positions (Figure 3.4 C). H3K9ac levels decreased when the plants were suffering from nitrogen depletion. At the distal promoter, there was a 93 % reduction of the acetylation level under nitrogen depletion. At the proximal promoter at position -200 bp there was a 77 % reduction under nitrogen depletion, while the beginning of the transcribed region, at position +10 bp relative to

the TIS, demonstrated a reduction of 90 % under nitrogen depletion. Therefore, nitrogen depletion showed a strong influence on the acetylation level at all three of the positions and not only on the distal promoter. This means, in contrast to the proximal promoter in maize, the proximal promoter in rice showed reduced acetylation levels under nitrogen depletion. This effect demonstrated a clear difference between transgenic rice and the donor species maize. At the other positions, maize and rice reacted similar.

Summarizing, the results obtained for H3K9ac in maize in the present thesis corresponded to what was already known for H3K9ac from previous studies (Offermann *et al.*, 2008). Notably, rice demonstrated a different reaction of acetylation levels at the proximal promoter in comparison to maize. Similarities between the two species were only observed at the distal promoter and at the beginning of the transcribed region. The mRNA level reacted similar in maize and rice, as it was reduced under nitrogen depletion in both species. This was accompanied by similarities at the distal promoter and the beginning of the transcribed region. The difference between the two species lay in the reaction at the proximal promoter in response to nitrogen depletion. Acetylation levels of H3K9 at the proximal promoter in maize were only dependent on illumination, but not on nitrogen availability. Therefore, they did not demonstrate a correlation with the transcriptional activity, which was measured via the mRNA level and was reduced as well under prolonged darkness, as under nitrogen depletion. In rice on the other hand, the acetylation correlated with the transcriptional activity at both positions, at the proximal promoter and at the distal promoter. In transgenic rice, a reduced mRNA level is accompanied by a reduced acetylation level at H3K9 at the proximal promoter, independent of whether it was provoked by prolonged darkness, or nitrogen depletion.

3.4.4 The H4K5ac level of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

In addition to the acetylation of H3K9ac, the modification level of H4K5ac has also been documented for nitrogen depleted plants and intact control plants of maize and transgenic rice expressing C4-*PEPC* (Figure 3.4 E).

The acetylation of lysine residue 5 of histone 4 was one of the lysine residues for which Offermann *et al.* (2008) evaluated the regulation under nitrogen depletion, as described above. Similar to the situation for H3K9ac, the results of said study revealed a different regulation of the distal promoter and the proximal promoter. While the distal promoter showed reduced acetylation levels under

nitrogen depletion, the acetylation levels at the proximal promoter did not react to nitrogen depletion. The same was observed for the beginning of the transcribed region (Offermann *et al.*, 2008).

In the present thesis, H4K5 acetylation levels were similar in nitrogen depleted maize and intact control plants of maize, at all three positions (Figure 3.4 E). Based on previous results, it was expected that the proximal promoter and the distal promoter should demonstrate a different regulation, like it was also expected for H3K9ac. Even if the results of H4K5ac in maize plants of the present thesis did not show this effect, the results of transgenic rice differed from what was expected for maize. Similar to H3K9ac, acetylation levels of H4K5 demonstrated a reduction in response to nitrogen depletion at all three of the representative positions. There was an 86 % decrease of H4K5ac under nitrogen depletion at the beginning of the transcribed region, at position +10 bp relative to the TIS. The distal promoter, at position -1050 bp showed a 52 % reduction of acetylation levels, while the proximal promoter, at position -200 bp relative to the TIS, showed a 55 % reduction of acetylation levels under the influence of nitrogen depletion (Figure 3.4 E).

To sum up, H4K5ac showed a different reaction towards nitrogen depletion in maize and transgenic rice, as there was no differential regulation of the proximal and the distal promoter in transgenic rice. The results of H4K5ac were, therefore, similar to H3K9ac. In maize, the acetylation level of H4K5 correlated with transcriptional activity only at the distal promoter. At the distal promoter H4K5ac (as well as H3K9ac) was reduced under prolonged darkness and under nitrogen depletion (Offermann *et al.*, 2008). H4K5ac at the proximal promoter on the other hand was only reduced in response to prolonged darkness, but not in response to nitrogen depletion, while the mRNA level was reduced in response to both stimuli (Offermann *et al.*, 2008). The situation in rice was different. In transgenic rice, both positions were characterized by a correlation between the mRNA level and the acetylation level of H4K5. Nevertheless, the behavior of the mRNA level in both species was similar (Figure 3.4 A). Notably, the reduction of the C4-*PEPC* mRNA in transgenic rice was stronger than in maize.

3.4.5 The H3K18ac level of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

A third histone acetylation has been evaluated in addition to H3K9ac and H4K5ac. H3K18ac has been documented for nitrogen depleted plants and intact control plants, for maize and transgenic rice expressing C4-*PEPC* (Figure 3.4 D).

As well as H3K9ac and H4K5ac, H3K18ac was one of the lysine residues for which Offermann *et al.* (2008) evaluated the regulation under nitrogen depletion, as described above. Similar to the situation for H3K9ac and H4K5ac, the results of said study revealed a different regulation of the distal promoter and the proximal promoter. While the distal promoter showed reduced acetylation levels under nitrogen depletion, the acetylation levels at the proximal promoter did not react to nitrogen depletion. The same was observed for the beginning of the transcribed region (Offermann *et al.*, 2008).

In the present thesis, H3K18ac showed similar levels in nitrogen depleted plants and intact control plants of maize at all of the three positions (Figure 3.4 D). Based on the results presented in previous studies, it should be expected that the acetylation level at the distal promoter would demonstrate a reduction under nitrogen depletion, while the proximal promoter should show constant acetylation levels in both situations. Therefore, the results corresponded to previous results only in the case of the proximal promoter, which showed no reduction of H3K18ac under nitrogen depletion. The distal promoter on the other hand differed from what was already known. Nevertheless, the effect that was observed for the acetylations of H3K9 and H4K5 in transgenic rice was also visible for H3K18ac. Again, the acetylation level corresponded to the expectations for maize at the distal promoter, as it demonstrated a strong reduction under nitrogen depletion. At position -1050 bp relative to the TIS, nitrogen depletion led to a 60 % decrease of H3K18ac. At the proximal promoter, the situation in transgenic rice differed from what was expected based on previous knowledge, as this position demonstrated decreased acetylation levels in nitrogen depleted plants, too. At position -200 bp relative to the TIS, there was a 45 % decrease, while the strongest decrease was observed at the beginning of the transcribed region. Here, nitrogen depletion led to a 91 % decrease (Figure 3.4 D).

Summarizing, this example again demonstrated a difference in the regulation of histone acetylations in transgenic rice In transgenic rice, all of the tested positions demonstrated a reduction of acetylation levels in response to nitrogen depletion. Therefore, all of the evaluated acetylations showed a different behavior in rice, as they were also reduced at the proximal promoter if the plant was suffering from nitrogen depletion. In contrast to the situation in maize, acetylation levels in transgenic rice at the distal and the proximal promoter both correlated with the mRNA level. For maize, it was expected that only the acetylation level at the distal promoter should show a correlation between the *PEPC* mRNA level and the H3K18ac level. The acetylation level of H3K18 at the proximal promoter on the other hand should only be dependent on illumination but was not expected to correlate with mRNA levels. In transgenic rice, this situation is different, as a correlation was detected at all of the representative positions.

3.4.6 The H3K4me3 level of C4-*PEPC* in maize and transgenic rice under the influence of nitrogen depletion

In addition to the three histone acetylations, the modification level of the trimethylation H3K4me3 has also been documented for nitrogen depleted plants and intact control plants, for maize and transgenic rice, expressing C4-*PEPC* (Figure 3.4 F).

Concerning the distribution of H3K4me3 over the gene, it was observed that the trimethylation was absent at the distal promoter. Neither intact control plants, nor nitrogen depleted plants showed strong signals at position -1050 bp relative to the TIS. This was observed for both species, in the donor plant maize, as well as in transgenic rice (Figure 3.4 F). Therefore, the results of the distribution of H3K4me3 in general corresponded to what was already known and reported in previous studies (Danker *et al.*, 2008; Offermann *et al.*, 2008). In contrast to the situation at the distal promoter, the proximal promoter and the beginning of the transcribed region showed clear signals for the trimethylation, for both experimental conditions and in both species. However, intact control plants of maize showed higher measurements at position -200 bp relative to the TIS, than at position +10 bp relative to the TIS. In nitrogen depleted maize on the other hand, the signal strength increased towards the transcribed region. It, therefore, demonstrated the expected distribution. The same was observed for H3K4me3 in transgenic rice. Concerning the modifications reaction towards nitrogen depletion however, it was not possible to detect differences in intact and nitrogen depleted plants in both species. Nitrogen depletion seemed to have no influence on the trimethylation status. A correlation to the transcriptional activity was, therefore, not visible for H3K4me3.

Summarizing, the results of H3K4me3 were similar for maize and rice, as well with regard to the distribution over the gene body, as with regard to the reaction to nitrogen depletion. There was no correlation with the mRNA level in both species.

4 Discussion

Due to worldwide population growth and a decrease in cultivation areas, the agricultural productivity will have to increase by a minimum of 60 % until 2050 (Sheehy and Ferrer, 2008; Kajala *et al.*, 2011) to meet worldwide nutritional needs. As common breeding approaches pursued today will scarcely result in an increase in crop yields, strong enough to meet the requirements that will have to be met in the future (Kajala *et al.*, 2011) transgenic approaches to introduce the C4-mechanism into C3-crops, might offer a possible solution for already existing nutritional problems. If genes that are necessary for a functional C4-photosynthesis are expressed in C3-crops, mechanisms ensuring the correct regulation of these genes might be of great importance. The ability to regulate C4-genes in a similar fashion in which they would be regulated in an actual C4-plant might, therefore, be important for a successful integration of C4-photosynthesis in C3-plants. Therefore, the question arises whether regulatory mechanisms already present in C3-plants are able to regulate the foreign C4-gene that is introduced into the C3-plant. This might help to ensure a correct regulation of C4-genes without the necessity to introduce the regulators that are responsible for the regulation of C4-genes in actual C4-plants. The subject of the present thesis was, therefore, the regulation of C4-*PEPC* and C4-*PPDK* of *Zea mays*, expressed in the C3-crop *Oryza sativa indica*. The results observed for C4-gene regulation in rice, have to be compared to what is already known for the correct regulation of said genes in the donor species maize. On the basis of this comparison, conclusions can be drawn concerning the feasibility of C4-gene regulation in C3-crops. For this purpose, the present thesis is referring to previous studies evaluating the behavior of C4-*PEPC* and C4-*PPDK* in response to different stimuli, including the influence of illumination, the influence of nitrogen depletion and the occurrence of a diurnal rhythm. In this context, both the mRNA level of the gene product and the chromatin status have been evaluated, in order to find out to which extend a correlation can be observed between the chromatin state and the mRNA level of C4-*PEPC* expressed in an actual C3-crop and in order to test whether C4-gene regulation is maintained in the transgenic plant. Each experimental setup included specimens of the donor species maize as comparison to the chromatin state and transcriptional activity in rice. The results are critically discussed, also with regard to their informative value for the function of the C4-genes at question in C3-crops like rice.

4.1 The transcriptional activity of C4-*PEPC* and C4-*PPDK* in response to illumination is correctly regulated in transgenic rice lineages and is accompanied by a similar modification pattern to that of the donor species maize

It is known that illumination is one of various stimuli which regulate C4-*PEPC* and C4-*PPDK* expression in maize plants. The influence of light strongly induces the transcriptional activity of both genes (Sheen, 1999; Horst, 2009; Heimann *et al.*, 2013). Therefore, the influence of illumination and prolonged darkness on transcriptional activity and the chromatin state was compared between the C4-plant maize and transgenic rice, expressing C4-*PEPC* or C4-*PPDK*, in order to find out whether they show similarities between the donor species *Zea mays* and transgenic specimens of the C3-crop *Oryza sativa indica*. The focus was set on the ability of the transgenic rice lines R1001 and R1002 expressing C4-*PEPC* and the transgenic rice lines R1007 and R1008, which are expressing maize C4-*PPDK*, to regulate the respective gene in the same way, in which it would be regulated in the donor species maize.

In this context, it is of special interest that the mRNA levels of both C4-*PEPC* and C4-*PPDK* showed a similar behavior in both transgenic rice lines and in the donor species *Zea mays*, which was used as a control. Both maize and transgenic rice demonstrated a reduction of more than 90 % of mRNA levels in plants exposed to prolonged darkness (3.1.1, Figure 3.1 A and 3.2.1, Figure 3.2 A). This reduction was observed in both transgenic rice lines and, therefore, seems to be independent of the exact integration site. On the other hand, illuminated plants of both species demonstrated light induction of C4-*PEPC* and C4-*PPDK*, characterized by increasing mRNA levels. Therefore, it was shown that with regard to light induction, the regulation of C4-*PEPC* and C4-*PPDK* transcription is not only functional in actual C4-plants, but also if it is expressed in the C3-crop rice. A strong light inducibility of C4-*PEPC* and C4-*PPDK* transcriptional activity has already been observed in previous studies (Danker *et al.*, 2008; Offermann *et al.*, 2008; Heimann *et al.*, 2013). The integration site might have caused a difference in the extent to which the C4-*PPDK* mRNA level was reduced, as the reduction was much stronger in R1008 in comparison to R1007 (Figure 3.2 A). Possible influences of the integration site are discussed further below.

On the basis of the correct up- and down-regulation of C4-*PEPC* transcriptional activity in transgenic rice in response to illumination, the question arose whether this effect is accompanied by the same histone modification pattern as in the regular C4-plant maize. Previous studies described a correlation between the acetylation of H3K9 and H4K5 and the transcriptional activity of C4-*PEPC* and C4-*PPDK* (Offermann *et al.*, 2008; Horst *et al.*, 2009; Heimann *et al.*, 2013). For other histone

modifications, like for example the acetylation of H3K18 and the trimethylation of H3K4, a correlation to the transcriptional activity and mRNA level could not be observed (Danker *et al.*, 2008; Offermann *et al.*, 2008). If the histone modification pattern that correlates with the regulation of C4-*PEPC* is maintained when the gene is expressed in rice, it should consequently be possible to observe light induction of H3K9ac and H4K5ac in transgenic rice, while the modifications H3K18ac and H3K4me3 should be uninfluenced by the light stimulus. It was, therefore, tested if and to what extent these histone modifications react to illumination or prolonged darkness in transgenic rice and whether they demonstrate a correlation with the mRNA level of C4-*PEPC* and C4-*PPDK*. H3K18ac was only included for C4-*PEPC*.

First of all, it was observed that the nucleosome density showed a correct distribution and reaction to illumination when the genes were expressed in the C3-plant *Oryza sativa indica* (3.1.2 and 3.2.2). This effect was also independent of the integration site, as in each case both transgenic rice lines showed a correct nucleosome density and reaction to illumination (Figure 3.1 B and Figure 3.2 B). Today, two mechanisms are known which influence the nucleosome occupancy. Apart from the intrinsic properties of a given sequence, which have an influence on the formation of nucleosomes, chromatin remodelers are also responsible for the formation and location of nucleosomes. These *trans*-acting factors are able to override the sequence-specific influences on nucleosome occupancy (Liu *et al.*, 2015). For *Arabidopsis thaliana* and *Oryza sativa*, it was for example possible to detect a negative correlation between the G/C content and the nucleosome occupancy. In *Arabidopsis thaliana*, the positioning of nucleosomes is further dependent on *trans*-acting factors which preferentially bind to certain motifs that can predominantly be found in genome regions characterized by a high G/C content (Liu *et al.*, 2015). However, as intrinsic properties based on the sequence play a role in the determination of nucleosome distribution, it is not surprising that the transgenic rice demonstrated a similar distribution of the nucleosome density to the one in maize. In this case, *trans*-acting factors do not seem to lead to differences in the nucleosome occupancy in both species and the pattern already known from maize is maintained in transgenic rice.

The results of the present thesis demonstrated light induction for H3K9ac. This effect was observed for both the donor species maize and transgenic rice expressing C4-*PEPC* (3.1.3, Figure 3.1 C) or C4-*PPDK* (1.1.1, Figure 3.2 C) respectively. According to previous studies, illumination provokes a 3 to 10-fold induction of H3K9 acetylation levels for C4-*PEPC*, depending on the position (Offermann *et al.*, 2008). The present results are, therefore, in accordance with previously described observations. Concerning the H3K9ac profile over the observed part of the gene body, an acetylation peak at position -200 bp relative to the TIS on C4-*PEPC* in illuminated maize plants is a typical and well-known feature of the genes regulation in response to illumination (Offermann *et al.*, 2008). This peak is also

present in transgenic rice, but shifted towards the beginning of the transcribed region (3.1.3). The fact that the peak was not detected at the same position in maize and transgenic rice might be due to the use of the maize hybrid Montello in the present thesis. This hybrid has been used here, as prior studies of the working group, to which the current results are mainly compared, were also based on the use of Montello. In contrast to inbred cultivars, Montello is derived from the crossing of the two homozygous parental maize lines FSIX and FPWW. It therefore, comprises two C4-*PEPC* alleles. Apart from those regular alleles derived from the parental lines, multiple copies of inactive pseudo-C4-*PEPC* copies have been detected (Dreesen, 2009). The influence of said pseudo-C4-*PEPC* copies might lead to the shift in the acetylation peak in Montello, as the inactive pseudo-gene is characterized by a high nucleosome density, but a low acetylation level. As the primers used in the present thesis are also able to bind to pseudo-C4-*PEPC*, the signals for the pseudo-gene might cover the peak at the beginning of the transcribed region (Dreesen, 2009), and might, therefore, lead to the observed shift. However, the shift of the peak at the proximal promoter in maize did not lead to differences in the reaction of the mRNA level to illumination or prolonged darkness in transgenic rice. Independent of the exact position of the acetylation peak in illuminated samples of maize and transgenic rice, there was an increase of the mRNA level under the influence of light. The regulation of H3K9ac was, therefore, correct in transgenic rice and showed the expected correlation with the mRNA level.

With regard to the distal promoter, the integration site in fact seemed to show a weak influence on the histone modifications distribution. It is known that position effects can influence the expression of transgenes depending on the site of integration (Feng *et al.*, 2001; Kumar and Fladung, 2001). For example, it has been reported that as well the neighboring plant DNA as the location on the chromosome can show a negative influence on the expression level of single copy transgenes (Kooter, Matzke and Meyer, 1999; Matzke *et al.*, 2000). Therefore, the structure of the integration site plays an important role in the determination of the expression level of a transgene (Kohli *et al.*, 2003). An example for this phenomenon is the repression of transgene expression in heterochromatic regions (Kooter, Matzke and Meyer, 1999). The influence of the surrounding DNA at the integration site has already been described by Pröls and Meyer in 1992. Said study evaluated the influence of different integration sites on the expression levels of transgenes in *Petunia hybrida* (Pröls and Meyer, 1992). The influence on the integration site was evaluated for the expression of the transgenes for the enzyme neomycin phosphotransferase II (*npt-II*), which confers kanamycin-resistance, and for the *Zea mays A1* gene, which is involved in the production of anthocyanin pigments (Pröls and Meyer, 1992). *A1* encodes a dihydroflavonol 4-reductase that is responsible for pigmentation (Reddy *et al.*, 1987). Therefore, the integration and successful expression becomes visible as specimens expressing *A1* show brick-red pigmentation (Meyer *et al.*, 1987). Three different

results have been reported for individual lines of *Petunia* that integrated the transgenes at different positions and showed strong, weak or no pigmentation. When the foreign fragment was integrated into a region of highly repetitive DNA, characterized by an A/T content of 39 % on the left and 57 % on the right site of the transgene, an inactivation of both genes was observed. Two other *Petunia* lines demonstrated expression of *npt-II* and *A1* at different intensities. An isolation of the insertion regions revealed methylation patterns that differed between the two lines. Specimens demonstrating strong pigmentation, due to high expression of *A1* and corresponding high expression levels of *npt-II*, were characterized by a hypomethylation at the integration site. Specimens demonstrating weak pigmentation and a lower transcriptional activity of *npt-II* on the other hand were characterized by high CG-methylation and additional CNG-methylation (Pröls and Meyer, 1992). It can, therefore, be concluded that the methylation pattern at the integration site is involved in the determination of transcriptional activity of the transgene. A connection between the transcriptional activity of a transgene and the methylation degree of the surrounding DNA of the integration site has been described by Jones *et al.* (1998), who observed the complex formation between MeCP2, which binds methylated DNA, and histone deacetylases (HDACs) (Jones *et al.*, 1998). A similar mechanism could be responsible for the acetylation profile of H3K9ac in R1002, as in comparison to the profile in R1001 it lacks an acetylation peak at the distal promoter (3.1.3). A possible explanation might be the recruitment of deacetylases due to the specific methylation pattern at the integration site of C4-*PEPC* in R1002. Notably, Offermann *et al.* (2008) demonstrated that the acetylation of H3K9 at the distal promoter of C4-*PEPC* in maize, in contrast to most of the other tested histone modifications, is not achieved by class I or class II HDACs. The influence of class I and class II HDACs was evaluated after Trichostatin A (TSA) treatment. TSA is an inhibitor of class I and class II HDACs (Yoshidas, 1990; Imai *et al.*, 2000). If a down-regulation of acetylation levels, for example in darkness (Offermann *et al.*, 2006), or in response to metabolic stimuli (Offermann *et al.*, 2008), is achieved by the activity of class I or class II HDACs, treatment with TSA consequently leads to an increase of acetylation levels. In this case, the activity of histone acetyltransferases (HATs) is also required at the specific site. In fact, acetylation levels were regulated by class I or class II HDACs, as TSA treatment led to an increase of acetylation levels even in plants that were illuminated. H3K9ac at the distal promoter of C4-*PEPC* did not show increased levels after TSA treatment. Therefore, it was concluded that H3K9ac levels at this position are not regulated by class I or class II HDAC activity, but instead by HAT activity (Offermann *et al.*, 2008). Concerning the regulation of H3K9ac at the distal promoter of C4-*PEPC* in transgenic rice, the question arises, whether a similar mechanism to the complex formation between MeCP2, which binds methylated DNA, and histone deacetylases (Jones *et al.*, 1998) might nevertheless be possible. In this case, the regulation of acetylation levels at the distal promoter of C4-*PEPC* would differ between maize and R1002. TSA treatment of R1002 might represent a suitable approach to

test whether class I or class II HDACs might be responsible for the regulation of H3K9ac levels at the distal promoter of C4-*PEPC* in transgenic rice, even if this is not the case in maize (Offermann *et al.*, 2008). In this case, TSA treatment would lead to increased H3K9ac levels at the distal promoter of C4-*PEPC* in R1002. Apart from this, another possible explanation might also concentrate on the responsible HAT which is required for H3K9ac. If the described HDACs are not responsible for H3K9ac down-regulation at the distal promoter in transgenic rice, reduced acetylation levels might also be due to a lack of HAT activity at the respective position in R1002. Offermann *et al.* (2008) were also able to observe differences in the recruitment of HATs to their site of activity, depending on the histone modification at question. In this context, HATs have been observed to be either recruited to a specific position, or not to be recruited to specific gene regions. If the responsible HAT is recruited to a specific gene region (in this case to the promoter and the coding region), acetylation is limited to these regions. This was still observed after inhibition of HDACs by TSA. In the case of H3K18ac and H3K14ac on the other hand, TSA treatment also led to an increase in acetylation levels at downstream regions and in subtelomeric regions used as negative control. It can thus be concluded that in the case of H3K18ac and H3K14ac, HATs are not specifically recruited (Offermann *et al.*, 2008).

In addition to the question whether class I and class II HDACs are also not responsible for H3K9ac at the distal promoter of C4-*PEPC* in transgenic rice, future experiments should also concentrate on the integration sites of the transgenes C4-*PEPC* and C4-*PPDK* in R1001 and R1002, or R1007 and R1008, respectively. If a functional C4-mechanism is introduced by the integration of C4-transgenes, the integration site plays a major effect on the transcriptional activity of these genes. For this purpose it would be necessary to identify and characterize the integration site. Furthermore, it should be isolated and evaluated with regard to its methylation degree to find out, which integration site would offer optimal conditions for C4-gene expression.

Notably, the difference in the acetylation pattern of H3K9 in R1002 did not lead to an impaired reaction of the mRNA level to illumination (3.1.1; Figure 3.1 A), as both R1001 and R1002 showed light induction. Concerning the distal promoter in maize, previous studies identified a peak at position -2000 bp relative to the TIS in (Offermann *et al.*, 2008). The importance of H3K9 acetylation peaks in the upstream region of numerous genes including C4-photosynthetic genes like C4-*PEPC* was also confirmed by Perduns *et al*. (2015). The described regulated secondary upstream peaks (R-SUPs) were distinct from peaks in the TIS area and were clearly regulated between the leaf base and the leaf blade. They demonstrated fold changes of up to 200 (in the case of C4-*PEPC*) between the leaf base and the leaf blade. These fold changes in acetylation levels between the base and the blade were further described to be associated with increasing transcriptional activity. For peaks that were

located in the TIS region (the region between the TIS and +1000 bp relative to the TIS), an up-regulation to this extend could not be observed. It was, therefore, suggested that R-SUPs play an important role in gene regulation with regard to the developmental gradient (Perduns, Horst-Niessen and Peterhansel, 2015). In the present thesis it was not possible to evaluate positions farther upstream than -1050 bp relative to the TIS, due to the limitations caused by the C4-*PEPC* construct introduced into rice. Nevertheless, the position -1050 bp relative to the TIS was used in the present thesis to evaluate the reaction of the distal promoter to illumination. As the promoter in general comprises a distal promoter beginning at position -600 bp relative to the TIS (Taniguchi *et al.*, 2000), the position measured in the present thesis is suitable to draw conclusions concerning the distal promoter. Nevertheless, if it is compared to the results presented in Offermann *et al.* (2008), it can only indicate a tendency, which is not as pronounced as the reaction at position -2000 bp. However, the acetylation of the promoter was in general similar to the situation in the donor species maize, if C4-*PEPC* was compared between the two species.

Concerning the distribution of H3K9ac in C4-*PPDK*, it was observed that acetylation levels increased between the upstream promoter and the beginning of the transcribed region (1.1.1, Figure 3.2 C). Independent of the position on the promoter, light inducibility was observed. These results were in accordance to previous data (Heimann *et al.*, 2013); (Horst, 2009). Both, the specific distribution as well as light inducibility of H3K9ac were maintained when the gene was expressed in rice.

The regulation of C4-*PEPC* and C4-*PPDK* mRNA in transgenic rice, therefore, correlated with an H3K9 acetylation pattern similar to the one in maize. Therefore, the regulation of both genes seemed to work in transgenic rice, as far as H3K9ac was concerned.

The second light inducible acetylation which was evaluated in transgenic rice, H4K5ac, demonstrated a correlation between the acetylation and the C4-*PEPC* mRNA level in both maize and R1001 (3.1.4, Figure 3.1 E). H4K5ac is known to show a strong reaction to illumination and to be positively correlated with the transcriptional activity (Offermann *et al.*, 2008; Heimann *et al.*, 2013). Like in the case of H3K9ac, previous studies demonstrated a 3 to 10-fold light induction for H4K5ac for C4-*PEPC* in maize depending on the respective position, although the acetylation was distributed more evenly than H3K9ac (Offermann *et al.*, 2008). Peaks for H4K5ac have been described for the proximal promoter and for the beginning of the transcribed region (Offermann *et al.*, 2008). Therefore, results of the present thesis corresponded to the data already known for H4K5ac in maize, except for the peak at the beginning of the transcribed region. This indicates that the establishment of H4K5 acetylation at the promoter is correctly performed in transgenic rice expressing C4-*PEPC*, as well. Notably, this could only be confirmed for R1001. The distribution of H4K5ac in R1002, as well as the reaction of the acetylation to illumination or prolonged darkness differed from the second transgenic

rice line. In R1002 it was not possible to observe light induction or a decrease of H4K5ac levels under prolonged darkness, respectively (Figure 3.1 E). Therefore, it seems as if the integration site might have an influence on the chromatin state if C4-*PEPC* is expressed in rice. This observation corresponds to the lack of H3K9 acetylation at the distal promoter of R1002 and would also fit to a possible recruitment of deacetylases (Jones *et al.*, 1998) due to the specific methylation pattern of the integration site in R1002. However, R1002 showed no correlation between H4K5ac and the mRNA level. It can therefore, be concluded that the difference in H4K5ac levels does not necessarily be mirrored in the transcriptional activity.

In transgenic rice, both C4-*PPDK* promoter positions showed decreased H4K5ac levels in R1007. A corresponding decrease at the distal promoter in R1008 could not be detected (3.2.4, Figure 3.2 E). At the proximal promoter on the other hand, both transgenic rice R1007 and R1008s reacted as expected on the basis of previous results of maize. A decrease of H4K5ac levels on C4-*PPDK* in response to prolonged darkness was observed at the distal and the proximal promoter (Horst, 2009; Heimann *et al.*, 2013). Notably, the fact that R1008 did not show a decreased acetylation level at the distal promoter did not lead to a weaker reduction in mRNA levels (Figure 3.2 E). In fact, the reduction of C4-*PPDK* mRNA upon darkness was even stronger in R1008 (90 % reduction in R1008 in comparison to 38 % reduction in R1007). This indicates that the constant acetylation level at the proximal promoter in R1008 does not lead to increased mRNA levels. A correlation between acetylation levels at the proximal promoter and the mRNA level can be observed in both R1007 and R1008.

The distribution of H4K5ac over the observed part of C4-*PPDK* in maize stayed constant in illuminated maize plants and did not show regions with increased levels of H4K5ac (Figure 3.2 E). It, therefore, differed from what was described by Heimann *et al.* (2013). In darkened maize on the contrary, it was possible to observe a general increase of H4K5ac levels from the distal promoter towards the beginning of the transcribed region. R1007 demonstrated a slight increase in acetylation levels for H4K5 between the distal promoter and the proximal promoter. It reached a maximum at position -300 bp relative to the TIS. Then the acetylation level decreased again between the proximal promoter and the beginning of the transcribed region. The distribution of H4K5ac in darkened specimens of R1007 was distributed more evenly and in general showed a lower level than the acetylation in illuminated plants. A similar distribution was observed for R1008. Both illuminated and darkened R1008 showed increasing H4K5ac levels between the distal promoter and position -300 bp relative to the TIS. It dropped again towards position -150 bp relative to the TIS.

Recent studies concentrating on maize, demonstrated an H4K5ac distribution that was similar to what was known for the distribution of H3K9ac on C4-*PPDK*. Dong *et al.* (2016) measured the

transcriptional activity of C4-*PPDK* and cytosolic isoforms, as well as the acetylation level of H4K5 in different organs in maize, including roots, stems, leaves and seeds. In this context, the acetylation level was evaluated at the positions -1420 bp, -850 bp and -180 bp relative to the TIS. Like H3K9ac, H4K5ac of C4-*PPDK* demonstrated increasing levels between the positions located further upstream and the proximal promoter at position -180 bp. Concerning the reaction of the acetylation level at said positions to the illumination status it was observed that H4K5ac reacted similar to H3K9ac. With regard to the studies of Dong *et al.* (2016), the results gained for the transcriptional activity and modification pattern of cytosolic, non-C4 isoforms of *PPDK* raise further questions. As already described in detail in 1.5 of the present thesis, *PPDK* expression is based on a dual promoter system which has been discovered in both maize and rice. Maize C4-*PPDK* shows great similarity with cytosolic *PPDK*, which represents the ancestral gene. Apart from this, the promoter of the cytosolic isoform is located within the coding region of C4-*PPDK* (Sheen, 1999). While the promoter of the cytosolic isoform shows ubiquitous activity, the C4-promoter is responsible for leaf-specific expression and the reaction towards illumination (Sheen, 1999). Therefore, the results of the mRNA and acetylation level of the cytosolic isoform, which are presented in Dong *et al.* (2016) might be influenced by signals measured for the C4-isoform of *PPDK*.

In the present thesis, the histone modification H3K18ac of C4-*PEPC* did not react to illumination, neither in maize nor in rice (3.1.5, Figure 3.1 D). Actually, the results of the experiments demonstrated an anti-correlation of H3K18ac to illumination in transgenic rice, which was also described by Horst (2009) for maize, but not in the aforementioned study by Offermann *et al.* (2008). The observed results corresponded to the expectations, as H3K18ac was included as example for an acetylation which is not inducible by light and, therefore, not associated with the mRNA level (Offermann *et al.*, 2008). It was also observed that modifications like H3K18ac, H3K14ac and H4K16ac, which are located closer to the histone body, demonstrated a different behavior than modifications located at the outside, which means a greater distance to the histone body. In contrast to the outermost lysine acetylations on histones H3 and H4 (H3K9ac and H4K5ac), they did not show light induction. It was, therefore, suggested that H3K18ac could contribute to a poised (which means a state ready for activation) and potentiated chromatin state, allowing rapid adaptations of the transcriptional activity (Offermann *et al.*, 2008). This hypothesis was in accordance with the previously described gradually progressing acetylation of histone 4 in *Arabidopsis*, beginning at lysine 16 and ending at lysine 5 (Earley *et al.*, 2007). In this case, H3K18 would be acetylated prior to H3K9. It would, therefore, already be acetylated before the outermost lysine acetylation H3K9ac is established in response to illumination. In Horst (2009), however, the hypothesis suggesting an influence of the position of the lysine in the histone side chain could not be confirmed. In contrast to what was postulated by said hypothesis, the distal promoter of C4-*PEPC* demonstrated light

dependent differences for the acetylation H3K23ac. The position of this histone acetylation on the side chain is also located in closer proximity to the histone body. Nevertheless, it demonstrated light inducibility. This was further underlined by the observations for the histone modifications H3K23 and H3K27 at the distal promoter of C4-*PPDK* and C4-*ME*, which were light inducible, too (Horst, 2009). Therefore, the position on the histone side chain and the distance to the actual histone body do not seem to determine whether a specific histone modification reacts to illumination. Independent of this question, H3K18ac did not react to illumination, in contrast to the two acetylations H3K9ac and H4K5ac. Therefore, a possible function of the more inward located modification H3K18ac in the creation of a poised chromatin state was suggested by Offermann *et al.* (2008). In this context, a poised chromatin state describes a situation in which the acetylation of H3K18ac is present and provides the basis for additional activating events, like H3K9 acetylation. The present results suggest that a possible function of H3K18ac in the establishment of a poised chromatin state might be conserved and functional, when the gene is transferred to the C3-crop rice. This would fit to the observation that the mRNA level reacted to light induction in both species, while H3K18ac did not show any differences.

In the present thesis, no light induction was observed for H3K4me3 levels in maize and both transgenic rice lines for C4-*PEPC* and C4-*PPDK* (1.1.1, Figure 3.1 F and 3.2.5, Figure 3.2 F), corresponding to the expectations based on previous studies (Danker *et al.*, 2008). These findings indicate that H3K4me3 still seemed to be able to perform its function in autonomous cell-type specific regulation (Danker *et al.*, 2008), when C4-genes are expressed in C3-crops. It might, therefore, indicate that the role of the trimethylation in developmental cell-type specific expression-patterns, which is independent of external stimuli, might be conserved and functional, when the genes are expressed in the C3-crop rice. This would also fit to the observation that the mRNA levels reacted to light induction in both species (Figure 3.1 A and Figure 3.2 A), while H3K4me3 did not show any differences. In this context, H3K4me3 would be responsible for a poised state that enables subsequent activation in the correct cell-type. In the case of C4-*PEPC* and C4-*PPDK*, this means in the mesophyll-cells. This would be of great importance, as it is one of the characteristics of C4-photosynthesis, which allow the primary fixation of CO_2 in the mesophyll and the subsequent establishment of increased CO_2 concentrations in the bundle sheath cells. It would, therefore, be interesting to investigate, whether cell-type specificity of C4-*PEPC* and C4-*PPDK* expression and the establishment of H3K4me3 are also conserved in transgenic rice. It was not possible yet to separate mesophyll cells and bundle sheath cells of rice. Future experiments should concentrate on this question to evaluate whether cell-type specificity for H3K4me3 is really given in transgenic rice.

Concerning the distribution of H3K4me3, an absence of the trimethylation at the distal promoter was observed for both genes and in both species (1.1.1, Figure 3.1 F and 3.2.5, Figure 3.2 F). In previous studies, H3K4me3 on C4-*PPDK* could not be detected at position -800 bp of the distal promoter (Heimann *et al.*, 2013). Absence of H3K4me3 was also described for the distal promoter of C4-*PEPC* (Danker *et al.*, 2008). This observation corresponded to what was already known for the characteristic distribution of H3K4me3, which usually peaks in the 5´region of gene sequences (Santos-Rosa *et al.*, 2002; Heintzman *et al.*, 2007; Heimann *et al.*, 2013).

Summarizing, all of the tested histone modifications demonstrated strong similarities in the donor species maize and in transgenic rice for both genes. Nevertheless, it has to be considered that the degree, to which the C4-*PPDK* mRNA level was reduced under prolonged darkness, differed in R1007 and R1008. In R1007, mRNA levels decreased slower than in R1008. Concerning C4-*PPDK* histone modifications, the only difference which was observed was the H4K5 acetylation of the distal promoter in R1008. Here, the acetylation level was not reduced under prolonged darkness. However, it is not probable that this difference lead to a different reaction of the mRNA level to illumination or prolonged darkness, as the modification is positively associated with mRNA levels, which contradicts a faster reduction in R1008 for C4-*PPDK* mRNA levels caused by the situation at the distal promoter. If differences occurred between R1001 and R1002, for example for the histone acetylation H3K9ac at the distal promoter of C4-*PEPC* or for the distribution and light inducibility of H4K5ac, these differences did also not lead to a different reaction of the C4-*PEPC* mRNA level to illumination or prolonged darkness in rice. Altogether, the transgenic rice was able to adapt C4-*PEPC* and C4-*PPDK* expression to illumination, independent of the integration site. The correct regulation of C4-*PEPC* and C4-*PPDK* was accompanied by a similar modification pattern in R1001 and maize, or in R1007, R1008 and maize, respectively. The fact that the regulation of mRNA levels worked in transgenic rice for both genes and in both rice lines demonstrates that the introduction of a functional C4-mechanism in rice would not require the additional introduction of C4-regulators. The present regulatory mechanisms in rice are already able to adapt the transcriptional activity to illumination or darkness.

4.2 The diurnal regulation of C4-*PEPC* transcriptional activity and modification patterns are conserved in transgenic rice

With regard to the transcriptional activity of C4-*PEPC* in transgenic rice, it was possible to observe a diurnal rhythm in both maize and transgenic rice. In R1001, the mRNA level decreased to 48 % after 16 h of illumination (3.3.1, Figure 3.3 A). The reduction in C4-*PEPC* mRNA throughout the day is even stronger than in the control species maize, for which 63 % of the mRNA level that was measured after 4 h of illumination was reached. This corresponded to the fact that the transcriptional activity of C4-*PEPC* is known to be not only regulated by external signals like illumination or nutrient availability, but is also dependent on an internal diurnal rhythm (Thomas *et al.*, 1990) under the control of circadian regulation (Horst *et al.*, 2009).

With regard to the control species maize, the nucleosome density did not change upon illumination or prolonged darkness. For 16 h illuminated plants, the nucleosome density was slightly higher than in 4 h illuminated plants. In transgenic rice, the values for darkened plants were higher than in illuminated plants. For 4 h and 16 h illuminated transgenic rice plants, there was no difference in the nucleosome density. The higher nucleosome density in darkened plants was not expected and did not correspond to previous observations (3.1.2). The differences are probably due to differences in the chromatin quality, which would explain the higher values for darkened plants.

In the present thesis, H3K9ac levels at C4-*PEPC* in R1001 demonstrated a functional diurnal rhythm and were, therefore, already reduced during the illumination period (3.3.2, Figure 3.3 C). This corresponded to the diurnal regulation of H3K9ac levels in the control species maize and demonstrated a correlation to the mRNA level which was also reduced while the plants were still illuminated. The same result was observed in case of the histone acetylation H4K5ac. Like in the case of H3K9ac and in accordance to previous data (Horst *et al.*, 2009), H4K5 acetylation demonstrated a clear reduction after 16 h of illumination, while the plants were still exposed to full illumination (3.3.3, Figure 3.3 E). This effect was conserved when the gene was expressed in rice. It has already been described previously that the diurnal rhythm of C4-*PEPC* transcriptional activity is accompanied by a diurnal rhythm in the maintenance of C4-*PEPC* histone acetylations (Horst *et al.*, 2009). For H3K9ac and H4K5ac, this effect was observed at both the distal and the proximal promoter. It can be concluded that the diurnal rhythm of the histone acetylation is still functional when the gene is expressed transgenic rice. This underlines that light is not the only stimulus which is responsible for acetylation events at the proximal promoter of C4-*PEPC.* It has been demonstrated that light alone is not sufficient for the establishment of acetylations, although illumination is a necessary prerequisite

for H3K9 and H4K5 acetylation. Illumination will only lead to increased acetylation levels, if the underlying diurnal rhythm of C4-*PEPC* allows light induction. Therefore, it was observed that acetylation levels are already reduced in the course of a diurnal rhythm, when there is still full illumination.

Concerning the presence of a diurnal rhythm of H3K18 acetylation, it was observed that the control plant maize did not show any differences between 4 h and 16 h illuminated plants in the present thesis (3.3.4, Figure 3.3 D). While Horst *et al*. (2009) described a reduction in H3K18ac levels in the distal promoter, beginning at position -1000 bp relative to the TIS and further upstream, it was not possible to detect this decrease neither in maize, nor in transgenic rice. This might be due to the limitations caused by the C4-*PEPC* construct introduced into rice in the present thesis. At this point it would have been interesting to evaluate if said reduction would be detectable further upstream than -1050 bp relative to the TIS. Nevertheless, concerning the proximal promoter and the beginning of the transcribed region, the results of the control species maize corresponded to the expectations and a diurnal regulation could not be observed for H3K18ac. This was also observed for transgenic rice, as far as the proximal promoter was concerned. For the beginning of the transcribed region on the other hand, rice demonstrated a reduction in H3K18 acetylation levels. This might correspond to the suggested role of H3K18ac in the establishment of a poised state of C4-*PEPC*. H3K18ac does not show any reaction to illumination, but demonstrated reduced acetylation levels at the distal promoter, when the plant was suffering from nitrogen depletion. In addition to this, the proximal promoter in transgenic rice demonstrated reduced H3K18ac levels after 16 h of illumination. Therefore, the modification might contribute to the establishment of a poised state of C4-*PEPC* as long as the nutrient availability and the diurnal rhythm allow it. Notably, it has to be considered that the anti-correlation of H3K18ac to light, which has been described previously by Horst *et al.* (2009) and which could also be observed in the present thesis, was not detected in rice at this point. In maize however, the anti-correlation was observed. A possible explanation might be a lower quality of the chromatin generated from darkened rice plants. A similar phenomenon was observed for H3K4me3 (see below).

Apart from the described histone acetylations, the light-independent histone trimethylation H3K4me3 has also been included in the present experiment. H3K4me3 represents an autonomous, cell-type specific modification (Danker *et al.*, 2008). In fact, H3K4me3 did not show reduced levels after 16 h of illumination in maize (3.3.5, Figure 3.3 F). This was also observed in the recipient species rice. Therefore, a correlation with the mRNA level throughout the time course could not be observed. This is in accordance to the fact that said modification is not inducible by illumination and presents a further indication for the maintenance of the cell-type specificity of H3K4me3 in

transgenic rice. As well as suggested for H3K18ac, H3K4me3 would in this case be responsible for the establishment of a poised chromatin state on the basis of which further activating histone modifications can occur. Notably, H3K4me3 in control plants which have been darkened for 48 h was slightly reduced in comparison to illuminated plants (Figure 3.3 F). This was not expected, as H3K4me3 is independent of illumination (Danker *et al.*, 2008), which has also been observed in previous results of the present thesis. A possible explanation might be a lower quality of the chromatin of darkened plants. This would also explain why H3K18ac did not show an anti-correlation to light, as described in previous results of the present thesis and in (Horst, 2009). However, concerning the role of H3K4me3, cell-type specificity cannot be confirmed in transgenic rice, as it was not possible to successfully isolate bundle sheath cells from R1001, yet. In further experiments it would, therefore, be of great importance to confirm this role by comparison to the H3K4me3 levels in bundle sheath cells of transgenic rice expressing C4-*PEPC*.

4.3 The transcriptional activity of C4-*PEPC* in response to nitrogen depletion is correctly regulated in transgenic rice but the modification pattern shows differences to the modification pattern of the donor species maize

Another important external stimulus which influences the transcriptional activity of C4-genes is the availability of nutrients. In this context, the effects of nitrogen depletion on the transcriptional activity and chromatin state of C4-*PEPC* have been studied in detail (Offermann *et al.*, 2008). Concerning the transcriptional rate of C4-*PEPC* under the influence of nitrogen depletion in the present thesis, there was a decrease in both maize and rice, which was stronger in rice (3.4.1, Figure 3.4 A). The transcriptional rate was reduced to a stronger degree (80 %) in Offermann *et al.* (2008). This might be due to the fact, that in the present thesis, mRNA levels were measured instead of hnRNA levels. Apart from this, the treatments differed in that in Offermann *et al.* (2008), the plants were transferred to the zeatin-solution or tap water respectively, after 4 h of illumination and remained in the solution for further 3 h. In the present thesis on the other hand, the plants were transferred to tap water after 1 h of illumination and remained in the solution for additional 3 h. The results of the zeatin-control demonstrated that the expression of C4-*PEPC* in transgenic rice was also dependent from cytokine signals indicating available nitrogen sources in the soil. Therefore, a functional C4-*PEPC* regulation in response to nitrogen availability or depletion could also be observed if the C4-gene is expressed in the C3-crop rice. Like in maize, this regulation took place in response to cytokinin signals (Sugiharto *et al.*, 1992). As the aim of the present thesis was to find out whether nitrogen depletion in general leads to a similar reaction of the transcriptional level and chromatin state in transgenic rice, crosslinking experiments have only been performed with intact control plants and plants suffering from nitrogen depletion. The aim was to inactivate the promoter and transcriptional activity of C4-*PEPC*, although the plants were still illuminated. It was, therefore, irrelevant, if the inhibition of transcriptional activity can be proven to be traced back to missing cytokinin signals. Specimens that have been treated with zeatin were, therefore, not included in the evaluation of the chromatin state. The inactivation of transcriptional activity is a necessary prerequisite to determine whether the regulation of C4-*PEPC* is due to the charge neutralization, or to the specific histone code. In the latter case, the histone modification at question would be observed independent of transcriptional activity and would, therefore, not show a correlation to the mRNA level. Thus, zeatin-treated plants were not required to answer this question and only the comparison of nitrogen-depleted plants and intact control plants was relevant.

In contrast to the very similar response of mRNA levels in maize and rice to nitrogen depletion, histone modifications demonstrated a different behavior in the two species. For maize, the

nucleosome density was similar in nitrogen depleted plants and intact control plants as well for the distal promoter, as for the proximal promoter and the beginning of the transcribed region (3.4.2). In rice, the distal promoter and the beginning of the transcribed region demonstrated an increased nucleosome density in response to nitrogen depletion. Concerning acetylation levels, the results obtained for H3K9ac in rice corresponded to what was already known for H3K9ac from previous studies only with regard to the distal promoter (Offermann *et al.*, 2008) (3.4.3). For the histone acetylations H4K5ac, H3K9ac and H3K18ac, Offermann *et al.* (2008) demonstrated a strong reaction of acetylation levels to nitrogen depletion, which correlated with a decrease in mRNA levels. In this context it is of great importance that a differential regulation of the distal and the proximal promoter was observed for these acetylations (Figure 4.2, A). Notably, the acetylation level at the proximal promoter stayed constant under nitrogen depletion, while the acetylation level at the distal promoter was reduced if the plant was suffering from nitrogen depletion. On the basis of these findings, Offermann *et al*. suggested a model which is characterized by different regulatory events at the proximal promoter and the distal promoter. This model described a regulation corresponding to the charge neutralization model for the distal promoter (Figure 4.2, A). This means that at the distal promoter, the histone modifications are established following the integration of the influencing signals (Figure 4.1, left). Therefore, they show a reaction to all tested stimuli, whereby the strength of this reaction was dependent on the dose of the integrated signals. The histone modifications which are set in response to the integrated stimuli are then supposed to regulate the transcriptional activity by their specific acetylation level. This is caused by a neutralization of the positive charge and, therefore, a weaker interaction between histones and the associated DNA (Offermann *et al.*, 2008; Horst, Heimann and Peterhansel, 2013). On the proximal promoter on the other hand, Offermann *et al.* (2008) observed not a reaction to all tested stimuli, but instead specific combinations of a stimulus and a corresponding histone modification (Figure 4.2, A) (Offermann *et al.*, 2008). The suggested model, therefore, described a regulation corresponding to the histone code model for the proximal promoter. This means that at the proximal promoter, the histone modifications themselves represent the level on which integration of information takes place (Figure 4.1, right). In contrast to the situation described by the charge neutralization model, the individual histone modifications are not regulated in response to an earlier integration of information, but are established in response to a specific stimulus. In the next step, the individual histone modifications provide a means of informational storage and for the integration of numerous signals. On this level, the histone modifications are recognized by transcription factors which regulate the transcriptional activity (Horst, Heimann and Peterhansel, 2013). The acetylation at the distal promoter, therefore, showed a correlation to the mRNA level, as information integration takes place already on a higher level, while the acetylation at the proximal promoter did not show such a correlation, as the specific

mRNA level results from the integration of the information stored by the individual histone modifications (Offermann *et al.*, 2008). Therefore, the regulatory level on which histone modifications are established is not directly correlated to the transcriptional activity. Instead, transcriptional activity is regulated by the integrated information of all involved histone modifications (Figure 4.1). It correlates with the integrated information, which means with the sum of the effects caused by the individual histone modifications and not with the single modifications themselves (Offermann *et al.*, 2008; Heimann *et al.*, 2013). Rice demonstrated a different reaction of acetylation levels at the proximal promoter in comparison to maize (Figure 4.2, B). This might indicate that the above-mentioned model suggested by Offermann *et al.* (2008), was not conserved in transgenic rice, as the proximal promoter seemed to be influenced by more than one external stimulus (3.4, 3.4.3, 3.4.4 and 3.4.5, Figure 3.4 C to F). Therefore, it was not possible to observe specific pairs of a certain stimulus and the corresponding histone modification. Instead several stimuli had an influence on histone modifications at the proximal promoter, too. This means that the integration of the information provided by the different stimuli already took place before a specific level of the histone modifications was established. Therefore, histone modifications at the proximal promoter in rice did not belong to the level of informational integration but instead followed the results of this integration processes. In contrast to the proximal promoter in maize, the proximal promoter in transgenic rice seemed to follow the charge neutralization model (Figure 4.1, left). Consequently, acetylation levels at the proximal promoter correlated with the mRNA level. As the acetylation levels are a direct result of informational integration at both promoter regions, it can be speculated that there will altogether be a stronger correlation between acetylation levels at the promoter and mRNA levels in transgenic rice. As several signals act on the acetylation levels at both promoter regions, there might be stronger variations in acetylation levels. This might result in a stronger reaction of C4-*PEPC* mRNA levels, which could in fact be observed in the present thesis. If rice demonstrated a regulation according to the histone code model at the proximal promoter, there would be less dependency between the individual lysine residues and the mRNA level, as integration would still have to take place. Instead, integration already took place before acetylation levels were adapted, which means that the dependency between the individual lysine residues and the mRNA level is higher. Due to the fact that histone acetylation is associated with actively transcribed genes (Kouzarides, 2007) the observed positive correlation with the mRNA level does not surprise. In transgenic rice suffering from nitrogen depletion, there was not just one position that demonstrated a strong reaction towards the stimulus. There was a decrease in acetylation levels of all of the tested histone acetylations, at both the distal and the proximal promoter. Therefore, the stronger reaction of the mRNA level in transgenic rice might be caused by the in total stronger reduction of acetylations, if the events at the distal and the proximal promoter are summed up.

In this context, the question rises, whether this phenomenon might influence the feasibility of a functional C4-mechanism in C3-plants. As changes in acetylation levels in transgenic rice lead to a stronger reaction of the mRNA level of C4-*PEPC*, the transcriptional activity might be less stable than in the case of an actual C4-plant. Instead it can be speculated that it would show a stronger dependency on external and internal influences and, therefore, show less stability in its expression level. Therefore, photosynthetic efficiency might vary in a C3-plant with a successfully introduced C4-mechanism and might show a stronger variation in response to the external influences. This is an important point, which has to be considered in future approaches to generate rice performing C4-photosynthesis.

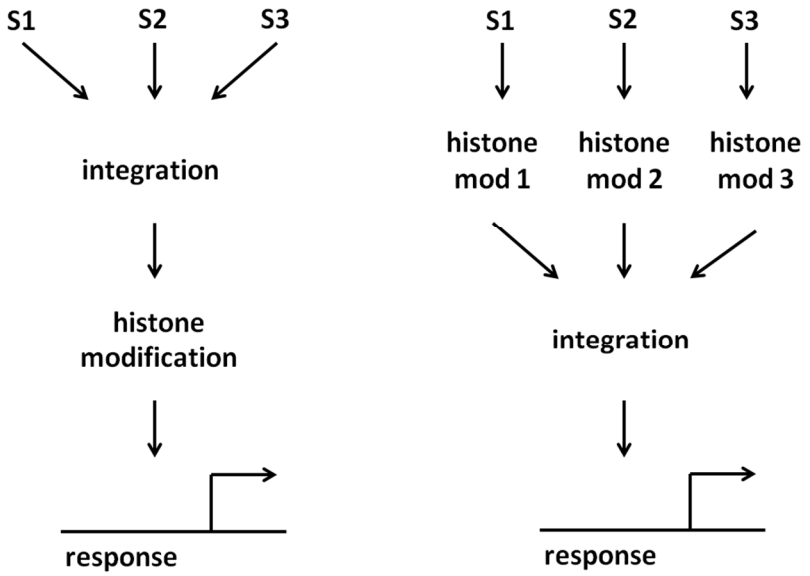

Figure 4.1: Comparison of the charge neutralization model and the histone code model of signal integration in the regulation of transcriptional activity. Two different models have been developed to explain the integration of external and internal signals received by a promoter, into a suitable response. The charge neutralization model (left), describes the integration of information on the first level. This integration process leads to the regulation of the response by the use of histone modifications, on the second level. The histone code model (right) considers histone modifications to represent the level of information integration. They are themselves regulated by external and internal signals. In the first case, a certain level of histone modifications is established in response to the signal integration, in the latter case, stimulus-modification pairs can be observed which are then integrated. The figure is taken from Horst, (2013). S1 = stimulus 1, S2 = stimulus 2, S3 = stimulus 3, mod 1 = modification 1, mod 2 = modification 2, mod 3 = modification 3.

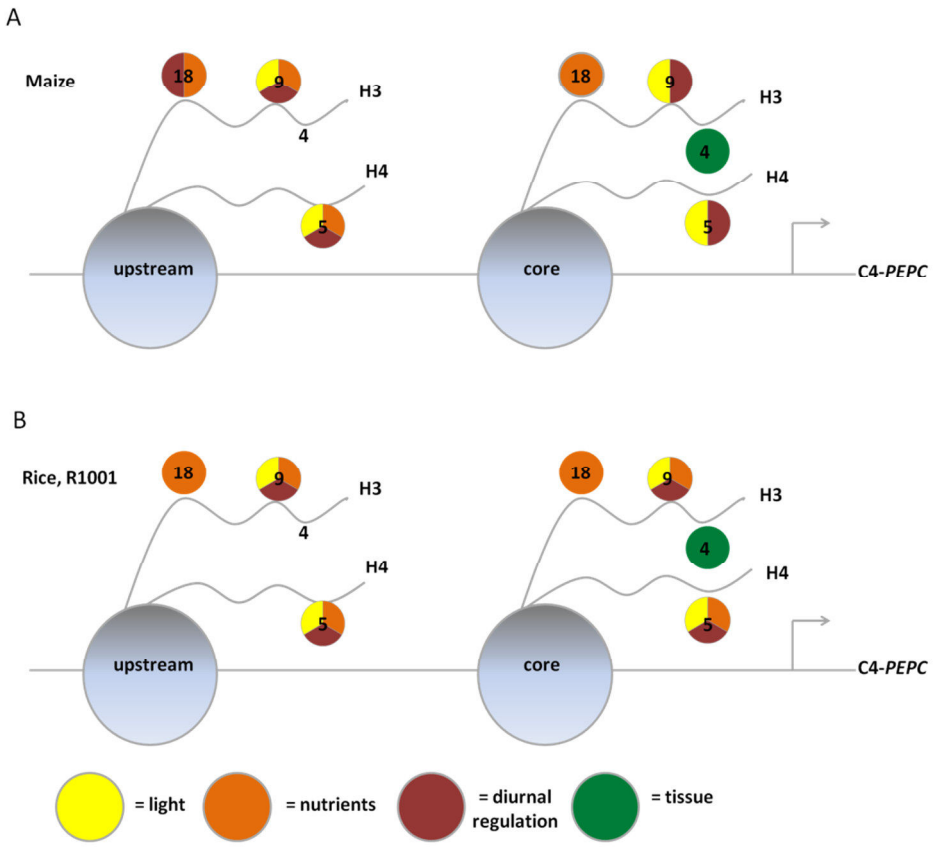

Figure 4.2: Schematic model of the function of the histone modifications tested in the present thesis in C4-*PEPC* gene regulation in the donor species maize and in transgenic rice. The tested lysine residues of the promoter region are presented with regard to their regulation by external and internal stimuli for the donor species maize and transgenic rice expressing C4-*PEPC*. The numbers represent the individual lysine residues located in the N-terminal tails of histone amino acid side chains for histones H3 and H4 in maize and transgenic rice. The tested stimuli are indicated by different colors, as defined in the figure. (A) Schematic model for the function of the histone modifications summarizing previously described regulatory effects of the stimuli tested in the present thesis in maize. (B) Schematic model for the function of histone modifications summarizing regulatory effects of the stimuli tested in the present thesis in transgenic rice expressing C4-*PEPC.* The figure is taken from Horst, Heimann and Peterhansel (2013) and adapted.

The described phenomenon could not only be observed in the case of H3K9ac, but also for H4K5ac (3.4.4) and H3K18ac (3.4.5, Figure 4.2). Similar to the situation in H3K9ac, both promoter regions were characterized by a correlation between the mRNA level and the acetylation level of H4K5 or H3K18 and, therefore, seemed to follow the charge neutralization model (Figure 3.4 D and E, Figure 4.1, left).

An interesting aspect in this context is the meaning for the suggested role of H3K18ac in the establishment of a poised or potentiated chromatin state that readily reacts to further stimuli (Offermann *et al.*, 2008). The fact that H3K18ac reacted to nitrogen depletion with a reduction which correlated with the mRNA level underlines a possible function in the maintenance of a poised chromatin state, ready for the up-regulation of transcriptional activity, as soon as further required acetylation events take place. This potentiated chromatin state is not maintained, when nitrogen is

not available any longer. In this case, illumination will not lead to increased mRNA levels, as the plant is suffering from nitrogen depletion. A function like this would be conserved if C4-genes are expressed in rice, as H3K18ac reacted to nitrogen depletion but not to illumination, exactly like in maize.

Concerning the behavior of the trimethylation H3K4me3 in transgenic rice, strong similarities were observed for the donor species maize and the recipient rice (3.4.6). For the modifications reaction towards nitrogen depletion, it was not possible to detect differences in intact and nitrogen depleted plants in both species. Nitrogen depletion had no influence on the trimethylation status (Figure 3.4 F). A correlation to the transcriptional activity was, therefore, not visible for H3K4me3. The same observation was made with regard to the influence of illumination on H3K4 trimethylation levels (1.1.1, Figure 3.1 F). These observations corresponded to the fact that H3K4me3 actually plays an important role in the determination of autonomous cell-type specificity and does not show reactions towards external signals (Danker *et al.*, 2008). The function of H3K4me3 seems to be maintained when C4-*PEPC* is expressed in rice. The fact that the modification is independent of the external stimulus nitrogen availability underlines that its function in cell-type specificity might be conserved in rice.

Summarizing, the regulation of C4-*PEPC* in rice was functional and altogether corresponded to what was previously described for the actual C4-plant maize, as far as the reduction of mRNA levels under nitrogen depletion was concerned. However, the observed differences in the regulation of acetylation levels at the distal promoter indicate that histone modifications at the proximal promoter of C4-*PEPC* follow the charge neutralization model instead of the histone code model. Due to the activating effect of acetylations on the transcriptional activity, this might be the reason for the stronger reduction of mRNA levels in transgenic rice. In this context, a stronger reaction of C4-genes expressed in rice to external influences seems probable and is an aspect which has to be considered in the generation of rice performing C4-photosynthesis.

4.4 Characteristics of C4-gene expression in transgenic rice

Summarizing, the expression of the photosynthetic genes C4-*PEPC* and C4-*PPDK* mirrored the characteristic of the respective gene expression in actual C4-plants. For both genes, light induction of transcriptional activity was in general possible and a corresponding reduction of mRNA levels was observed when the plants were kept in prolonged darkness (3.1.1 and 3.2.1). This corresponds to recent observations by Li *et al.* (2017), who documented tissue-specific expression patterns of C4-homologs in *Populus* hybrids. The C3-plant demonstrated a correlation of H3K9 and H4K5 acetylation levels with the tissue-dependent expression pattern of the C4-homologs. TSA treatment led to a slight increase in the acetylation levels of H3K9 and H4K5. This was observed in all tissues, at the proximal promoter of nearly all of the tested C4-homologs. This demonstrates that the regulation of acetylation levels was dependent on HDACs. It was hypothesized that C3-plants in general possess similar regulatory mechanisms to the ones of C4-plants and that these mechanisms were recruited during C4-evolution (Li *et al.*, 2017). Apart from a correct regulation of the transcriptional activity of C4-*PEPC* and C4-*PPDK* in transgenic rice, the regulation of histone modifications in R1001 demonstrated great similarity to the regulation of the corresponding histone modifications in the donor species maize. However, it seemed as if the integration site had in part an influence on the light inducibility and profile of the tested histone acetylations, as the results of R1002 and R1008 differed from the observations for R1001 and R1007 (Figure 3.1 C and Figure 3.2 E). It is known that the characteristics of the integration site can have a major influence on the transcriptional activity of a transgene, as described in detail in 4.1 (Pröls and Meyer, 1992). However, these differences in acetylation levels did not lead to differences in the induction or decrease of mRNA levels. Therefore, it can be concluded that the sum of activating and repressing modification events in transgenic rice was still able to regulate the mRNA level in the same way in which it would be regulated in the donor species maize. Concerning the influence of nitrogen depletion and a diurnal regulation on C4-*PEPC* expression, transgenic rice was able to adapt the mRNA level in the same way as maize, although the reduction of mRNA levels under nitrogen depletion was much stronger in rice than in maize.

The evaluation of the chromatin state in response to nitrogen depletion however revealed a major difference between the two species which might contribute to the different degree of mRNA level reduction in the two species (Figure 3.4 A). As already described in detail in 4.3, Offermann *et al.* (2008) suggested a model which is characterized by different regulatory events at the proximal promoter and the distal promoter. This model described a regulation corresponding to the charge neutralization model for the distal promoter and a regulation corresponding to the histone code model at the proximal promoter. In transgenic rice, both the distal and the proximal promoter

reacted to nitrogen depletion with a reduction in acetylation levels at H4K5, H3K9 and H3K18 (Figure 3.4 C, D and E). Therefore, the regulation of both promoter regions corresponded to the charge neutralization model, as far as transgenic rice is concerned. As mentioned above, this difference in acetylation levels was accompanied by a stronger reduction of mRNA levels in nitrogen depleted transgenic rice, in comparison to nitrogen depleted maize. As already explained in 4.3 the stronger reaction of the mRNA level in transgenic rice might be caused by the in total stronger reduction of acetylations, if the events at the distal and the proximal promoter are summed up. Therefore, stronger reaction of C4-genes expressed in rice to external influences seems probable and has to be considered in the generation of rice performing C4-photosynthesis.

In this context it was also observed that the histone acetylation H3K18ac, which is not inducible by illumination, reacted to nitrogen depletion with a clear reduction at all tested positions in transgenic rice (Figure 3.4 D). Due to the fact that H3K18ac is not light-inducible, Offermann *et al.* (2008) suggested a possible role for H3K18ac in the establishment of a poised chromatin state that readily reacts to further stimuli, like illumination. If this holds true, H3K18 is acetylated as long as a sufficient amount of nitrogen is provided and, as far as the proximal promoter in rice is concerned, during the correct time-period throughout the diurnal rhythm. A further up-regulation of mRNA levels could then be achieved by light induction of H3K9ac and H4K5ac and during the correct time-period of the diurnal rhythm. Such a role of H3K18ac seems to be conserved and functional, if C4-*PEPC* is expressed in rice.

Concerning the establishment of acetylation events in transgenic rice expressing the C4-genes *PEPC* and *PPDK*, it can be concluded that the present rice histone acetyltransferases (HATs) and histone deacetylases (HDACs) are probably in part recruited in a similar fashion than the maize equivalents. Concerning illuminated leaves, it has been demonstrated that the acetylation levels of C4-*PEPC* in the donor species maize are regulated by the activity of class I and class II HDACs (Offermann *et al.*, 2008). Trichostatin A (TSA), which is known to inhibit class I and class II HDACs (Yoshidas, 1990; Imai *et al.*, 2000) has been used to generate increased acetylation levels of C4-*PEPC* in illuminated maize leaves. Notably, this effect was especially pronounced at the distal promoter, while the increase at the proximal promoter was weaker. H3K9ac represented an exception, as TSA treatment did not lead to increased acetylation levels at the distal promoter (Offermann *et al.*, 2008). This means that class I or class II HDACs are not responsible for the regulation of H3K9ac levels at the distal promoter of C4-*PEPC*, but that the activity of the corresponding HAT regulates H3K9ac levels at this position. A reduction of H3K9ac at the distal promoter is, therefore, caused by reduced HAT-activity and not by HDAC activity. However, as described in detail in 4.1, it might still be possible that class I and class II HDACs are responsible for H3K9ac regulation at the distal promoter of transgenic rice, even if they

are not responsible for this histone modification at the distal promoter in maize. However, a difference in HAT recruitment might also be responsible for differences at the distal promoter of R1002. In this case, the integration site might influence the recruitment of HATs and HDACs, as described in 4.1. At this point, the regulation of acetylation levels by class I or class II HDACs in maize is also interesting with regard to the different response to nitrogen depletion at the proximal promoter in transgenic rice. In this context, Offermann *et al.* (2008) observed that metabolic stimuli were able to reduce increased acetylation levels after TSA treatment. Except for H3K9ac, deoxygenase treatment or nitrogen depletion, led to unchanged levels of acetylation in TSA treated plants. TSA inhibited the class I or class II HDACs which were responsible for the down-regulation of acetylation levels. Therefore, the levels of acetylation stayed constant and were not down-regulated in response to the applied metabolic stimuli. H3K9ac on the contrary was reduced at the distal promoter. It could therefore, be concluded that HDACs were not responsible for this regulatory process, as otherwise there would not have been a down-regulation after TSA induced inactivation of HDACs. Instead, H3K9ac levels at the distal promoter were suggested to be regulated by the activity of HATs, as mentioned above. The HAT which is responsible for the establishment of H3K9ac levels at the distal promoter of C4-*PEPC*, as long as the plant is illuminated and as long as there are no repressing metabolic influences, demonstrated reduced activity under nitrogen depletion or deoxyglucose treatment. Therefore, the acetylation of H3K9ac at the distal promoter of C4-*PEPC* is regulated by HAT activity (Offermann *et al.*, 2008). The situation in rice was different. Transgenic rice demonstrated reduced acetylation levels at both the distal and the proximal promoter in plants suffering from nitrogen depletion (3.4.3, 3.4.4 and 3.4.5). In maize, metabolic stimuli were not able to inhibit the increase in acetylation levels that was achieved by TSA treatment, at the proximal promoter. Therefore, it was concluded that metabolic repression did not lead to increased HDAC activity at the proximal promoter. Consequently, acetylation levels were not reduced upon metabolic repression (Offermann *et al.*, 2008). This was different in rice. Here, nitrogen depletion did in fact lead to a decrease in acetylation levels at the proximal promoter. This was observed for H3K9ac, H4K5ac and H3K18ac (Figure 3.4 C, D and E). This decrease might be due to an increased HDAC activity in response to nitrogen depletion (similar to processes for the regulation of H4 acetylation at the distal promoter in maize), or to reduced HAT activity (similar to processes for the regulation of H3K9 at the distal promoter in maize). In this context it would be interesting to determine which rice HATs and HDACs are responsible for the regulation of acetylation levels on C4-genes expressed in the recipient species. By the use of the described TSA treatment, it would be possible to evaluate the role of HDACs in the establishment of the expected acetylation pattern of C4-genes in transgenic rice. HDACs are highly conserved in yeast, animals and plants, except for the plant-specific HD2 family (Lusser *et al.*, 1997; Pandey *et al.*, 2002; Ding *et al.*, 2012). Apart from this plant-specific family, the

RPD3, HDA1, and SIR2 families have been described. In rice, the function of most HDACs is still unclear (Ding *et al.*, 2012). Using the experimental approach of Offermann *et al.* (2008) in transgenic rice would render clear whether HDACs are also responsible for the regulation of acetylation levels on C4-genes in transgenic rice, as in this case, the transgenic C3-crop would also demonstrate increased acetylation levels after TSA treatment. In this case, it could be concluded that the increased acetylation levels that can be observed for C4-genes in illuminated leaves of transgenic rice, are also due to the repression of a HDAC by illumination. Also, it could be tested, whether H3K9ac at the distal promoter of C4-*PEPC* in transgenic rice is regulated by HDAC or HAT activity. Apart from HDACs, eight HATs which can be divided into four groups have been identified in rice. These are HAC701, HAC703 and HAC704 of the CBP family, HAF701 of the $TAF_{II}250$ family, HAG702, HAG703 and HAG704 of the GNAT family and HAM701 of the MYST family (Liu *et al.*, 2012). Sequence analyses revealed strong similarities between rice HATs and their homologs in maize, concerning the protein sequence, conserved domains and 3D models. Apart from this, a constitutive expression of all tested rice HATs was reported (Liu *et al.*, 2012). In this context, ChIP experiments could be performed in order to find out, whether one of the described HATs interacts with C4-genes expressed in rice and might, therefore, be responsible for acetylation events at the tested histone lysine residues.

In addition to this, it seems probable that a further factor which regulates the acetylation level in the context of a diurnal rhythm and which is already functional while the leave is still illuminated (Horst *et al.*, 2009), is also present in transgenic rice (3.3). This would explain the maintained diurnal rhythm of histone acetylations of C4-*PEPC* in transgenic rice. A possible regulatory factor might be hexose availability, which has a strong impact on C4-*PEPC* regulation (Jang and Sheen, 1994; Suzuki *et al.*, 1994). It has been demonstrated that 2-deoxyglucose (DOG) is able to mimic the availability of hexose in the cell (Jang and Sheen, 1994). Offermann *et al.* (2008) were actually able to observe reduced hnRNA levels for C4-*PEPC* upon DOG treatment, although not to the same extend as for nitrogen depletion. Similar to the reaction under nitrogen depletion, the proximal promoter did not show reduced acetylation levels after DOG treatment, while acetylation levels were reduced at the distal promoter. The reduction of acetylation levels at the distal promoter was dependent on DOG-concentration. Like in the case of nitrogen depletion, there was a differential regulation of the two promoter regions in maize (Offermann *et al.*, 2008). As this differential regulation could not be observed in transgenic rice, it would be interesting to investigate whether this holds also true for the effect of hexose availability. Therefore, transgenic rice could be treated with DOG, to evaluate if the regulation of the proximal promoter differs from the regulation of the distal promoter, or if similar to the situation in nitrogen depleted plants, the regulation differs from the regulation of acetylation events in maize. Concerning diurnal regulation, the reduction of transcriptional activity and histone

acetylations after 16 h of illumination might be caused by the increasing hexose availability throughout the day. However, this would only explain the reduction of histone acetylation at the distal promoter in maize. In this case, high hexose availability might increase HDAC activity, thus leading to a reduction in acetylation levels at the distal promoter. Notably, the acetylation levels at the proximal promoter were also reduced throughout the day in both maize and transgenic rice. This could not be explained by increasing hexose levels, at least not in maize. At the proximal promoter, increased hexose availability did not lead to reduced acetylation levels (Offermann *et al.*, 2008) and would, therefore, not be suitable to explain the reduction in acetylation levels throughout the day. The fact that this independence of the proximal promoter was observed in maize does not necessarily mean that the situation is similar in transgenic rice. As in the case of the influence of nitrogen depletion, the proximal promoter in transgenic rice might show a different regulation than the proximal promoter in maize.

H3K4me3 is supposed to play an important role in the formation of a poised chromatin state. It is independent of illumination and takes part in autonomous cell-type specific regulation (Danker *et al.*, 2008). As these characteristics of H3K4me3 could also be observed in transgenic rice (1.1.1, 3.2.5, 3.3.5 and 3.4.6), the function of H3K4me3 seems to be conserved and in transgenic rice as well. Lysine residues are methylated by histone lysine methyltransferases (HKMTs), most of which catalyze the reaction via a SET domain harboring the enzymatic activity. HKMTs transfer a methyl group derived from S-adenosylmethionine (SAM) to the ε-amino group of the respective lysine residue. The HKMTs are characterized by a high specificity for a given lysine and the degree to which it is methylated. The action of HKMTs is reversed by histone demethylases (Grillo and Colombatto, 2005; Nelissen *et al.*, 2007; Bannister and Kouzarides, 2011). The SET domain which is responsible for the enzymatic activity is conserved among different species (Zhang and Reinberg, 2001; Feng *et al.*, 2002). Recently, the methyltransferase SET DOMAIN GROUP 701 (SDG701) has been reported in rice. This methyltransferase is responsible for the specific methylation of H3K4 and takes part in the regulation of developmental processes (Liu *et al.*, 2017). As the trimethylation of H3K4me3 in transgenic rice demonstrated the same characteristics as in the donor species maize, it can be concluded that methyltransferases in rice are recruited in a similar way as in maize. SDG701 might be a suitable candidate for a further analysis of H3K4 methylation on C4-*PEPC* in transgenic rice. A possible approach might be to perform crosslinking experiments and subsequent ChIP to investigate, whether SDG701 is associated with the methylation of C4-*PEPC* in transgenic rice and if it is, whether its association with the modification is also independent of illumination. In this context, it would be of great value, to develop an experimental approach that allows the isolation of bundle sheath cells from the C3-crop rice, to test whether the mesophyll-specificity is also given in the transgenic recipient species.

The aim of the present thesis was to evaluate if transgenic C3-plants expressing C4-genes would be able to regulate the transcriptional activity and corresponding histone modifications of these genes in a similar manner to their regulation in an actual C4-plant. A correct regulation of C4-genes might be important for the establishment of a functional C4-mechanism in C3-crops like rice. To achieve a level of regulation that ensures the correct functionality of C4-genes in rice, the regulatory mechanisms already present in C3-plants might be suitable. The results of the present thesis demonstrated that the transcriptional activity of photosynthetic C4-genes expressed in rice, maintains the ability to adapt to different stimuli in a similar fashion as in the donor species maize. This was confirmed for light inducibility of the photosynthetic genes C4-*PEPC* and C4-*PPDK*, for the response of C4-*PEPC* to nitrogen availability and for the diurnal rhythm of C4-*PEPC.* A first conclusion is, therefore, that with regard to the tested stimuli and to the regulation throughout the day, the correct regulation seems to be maintained under expression in a C3-crop. However, while the transcriptional activity demonstrated a similar behavior than in maize, the regulation of histone modifications differed in part. For example, the differential regulation of the distal and the proximal promoter of C4-*PEPC* in response to metabolic stimuli could not be confirmed for rice. In rice, both promoter positions demonstrated a behavior, which can be described by the characteristics of the charge neutralization model. This might possibly lead to stronger reactions towards changing metabolic conditions, as acetylation levels show a direct positive correlation with mRNA levels. It can be speculated that the regulation of C4-photosynthesis engineered into transgenic rice might, therefore, be more vulnerable to suboptimal conditions like nitrogen starvation. In general, the results indicate that the regulators already present in C3-plants seem to be able to regulate most of the histone modifications in a similar way to the regulation in the donor species maize. However, the role of HATs and HDACs might differ. This has to be determined by TSA treatment, as already described in detail. In this context however, the regulation of histone modifications at the proximal promoter in rice seems to differ from the regulation in maize, as far as metabolic stimuli are concerned, leading to a stronger reaction in the mRNA level. However, these differences do not seem to impair the possibility of a functional C4-mechanism in rice, as the available regulators are in general able to ensure the correct adaptation of transcriptional activity to environmental and internal signals. Therefore, it might be sufficient to express the key enzymes of C4-photosynthesis in C3-crops like rice, without introducing the corresponding C4-regulators as well. This demonstrates that C3-crops like rice in fact seem to be able to regulate C4-genes similar to their regulation in C4-species.

4.5 Outlook on future experiments

Based on the reflections presented above, there are at least three important points which should be included in future experiments. First of all, the specific characteristics of the integration sites in different transgenic rice lines should be analyzed, as described in 4.1. Apart from this, the isolation of rice bundle sheath cells would be of great value for the evaluation of a possible cell-type specific expression of C4-genes in transgenic rice and their correlation with cell-type specificity of associated histone modifications, like H3K4me3. Furthermore, the identity of modifying enzymes in rice should be examined, to find out which factors are responsible for the expected modification pattern of C4-genes in transgenic rice.

However, if the possibilities of the introduction of a functional C4-mechanism in a C3-crop like rice are evaluated, C4-*PEPC* and C4-*PPDK* are not the only photosynthetic genes which should be analyzed in detail. Further important members of the C4-mechanism are carbonic anhydrase (C4-*CA*) in mesophyll cells, as well as NAD(P)-malic enzyme (C4-*ME*), phosphoenolpyruvate carboxykinase (C4-*PEPCK*) and rubisco in bundle sheath cells (Heimann *et al.*, 2013). The influence of light on H3K9, H4K5 and H3K18 acetylation has been studied in detail for these C4-genes. In general, all of these genes have been demonstrated to be light inducible, which was accompanied by induced acetylation levels of H3K9 and H4K5 at the distal and the proximal promoter. H3K18ac on the other hand was not induced by illumination, but remained constant for all genes except C4-*PEPCK*, which showed a weak induction (Heimann *et al.*, 2013). Apart from this, a cell-type specific trimethylation pattern of H3K4 was observed for all genes except rubisco. In this context, the H3K4me3 level was high in the cell-type in which the corresponding gene can be expressed (Heimann *et al.*, 2013). To find out whether a correct regulation is possible for all genes that are essential for the C4-photosynthetic mechanism, transgenic rice expressing said genes should be used to evaluate the modification patterns and the regulation of the transcriptional activity of these C4-genes, too. However, it has to be considered that the contribution of both mesophyll and bundle sheath cells is an important aspect of C4-photosynthesis. Even if it will be possible, to express the above-mentioned key-enzymes of C4-photosynthesis in C3-crops in a correctly regulated manner, the establishment of a Kranz anatomy like morphology in C3-leaves is one of the most important goals. Therefore, a further important step in engineering rice performing C4-photosynthesis might be the introduction and correct regulation of genes responsible for Kranz anatomy (Ku *et al.*, 2000). In this context, however, the introduction of a simplified C4-mechanism might represent an alternative. The aquatic plant *Hydrilla verticillata* for example is able to adapt to low CO_2 concentrations by performing a simplified C4-mechanism as alternative to C3-photosynthesis (Bowes and Salvucci, 1984, 1989; Ku *et al.*, 2001). The facultative

C4-plant does not require Kranz anatomy for this shift in photosynthetic mechanisms. In this case, C4-photosynthesis takes place in a single cell and is not characterized by the contribution of two distinct cell-types. Instead, the cell-compartments are responsible for establishing a higher CO_2 concentration around RUBISCO. This is achieved by the fixation of inorganic carbon in acid malate in the cytoplasm. This step is performed by *PEPC*. In the chloroplast, CO_2 is set free again, as malate is decarboxylated by NADP-ME. Consequently, CO_2 concentrations are increased around RUBISCO, which is also located in the chloroplast and photorespiration can be overcome (Bowes and Salvucci, 1989; Reiskind *et al.*, 1997; Ku *et al.*, 2001). Immunolocalization studies revealed that maize PEPC is expressed in the cytosol of transgenic rice. NADP-ME and PPDK on the other hand were observed in the chloroplast, indicating that the regulatory mechanisms present in rice are able to maintain the enzymes activity in the corresponding cell-organelle. Therefore, a single-cell C4-mechanism, like in the case of *Hydrilla verticillata*, might represent an alternative approach for the introduction of C4-photosynthesis in rice (Ku *et al.*, 1999, 2001). Apart from this, the influence of further environmental and internal influences might represent interesting working fields. For example, the reduction of C4-*PEPC* transcriptional activity and corresponding acetylation levels in response to hexose accumulation should be evaluated in transgenic rice as well.

The results of the present thesis help to answer the question, to what extend it is possible to introduce a functional C4-mechanism into C3-crops like rice, if functionality also depends on the histone modification profile. The results give evidence, that the correct regulation of C4-photosynthesis genes might be achieved in rice, even without introducing further regulators. Apart from this, the present results underline that a functional regulation of gene products taking part in the C4-mechanism is not dependent on an exact copy of the modification state in the donor species maize. The functionality of the genes still seems to be given, if there are differences in the exact modification pattern, or in the role the individual regulators play in rice and maize. However, the differences in modification patterns might also lead to stronger reactions of transgenic rice performing C4-photosynthesis, to suboptimal conditions. This is of great importance, as this factor has to be considered if the C4-mechanism shall be introduced in rice. Altogether the present results indicate that the C3-crop rice in fact seems to be able to regulate C4-genes in a similar way to their regulation in an actual C4-species. Therefore, the present results provide valuable information for the challenge to integrate a functional C4-mechanism in C3-crops like rice, which might be an important step towards the production of C3-crops with increased productivity. C3-crops which have been improved by the introduction of a functional C4-mechanism might contribute greatly to the management of increasing nutritional problems worldwide. We have now seen, that the approach to integrate the C4-mechanism into C3-crops, might offer a realistic possibility to ameliorate C3-crop productivity in the desired way, to face and solve these problems.

5 Bibliography

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6 Appendix

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Curriculum vitae

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