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- Pflanzenernährung -

Ascorbate Metabolism in Rice (*Oryza sativa* L.) and its Implications for Tolerance against Zinc Deficiency

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Printed articles:

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Contents

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Abbreviations

AAS	Atomic absorption spectrometry
AO	Ascorbate oxidase
APX	Ascorbate peroxidase
AQC	6-aminoquinolyl-N-hydroxysuccinimidylcarbamat
AsA	Ascorbic acid
BHT	2,6-di-tert-butyl-4-methylphenol
CA	Carbonic anhydrase
CAT	Catalase
DAB	3,3`-diaminobenzidine
DAT	Days after transplanting
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbic acid reductase
DJ	Dongjin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GGP	GDP-L-galactose phosphorylase
GME	GDP-mannose-3,5-epimerase
GR	Glutathione reductase
GSH	Reduced glutathione
H_2DCF-DA	Dihydrodichlorofluoresceindiacetate
H_2O_2	Hydrogen peroxide

LBS	Leaf bronzing score
MDA	Malondialdehyde
MDHA	Monodehydroascorbic acid
MDHAR	Monodehydroascorbate reductase
MIOX	*Myo*-inositol oxygenase
MPA	Metaphosphoric acid
NB	Nipponbare
NEM	N-Ethylmaleimide
NPQ	Non-photochemical quenching
PI	Propidiume iodide
PMI	Phosphomannose isomerase
PPFD	Photosynthetic photon flux density
PS	Photosystem
QTL	Quantitative trait loci
ROS	Reactive oxygen species
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
SOD	Superoxide dismutase
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TKW	Thousand kernel weight
Zn	Zinc

Summary

Rice is one of the most important staple crops worldwide and a growing world population will lead to an increasing demand for food in the upcoming years. Severe yield losses are threatening food security, caused by abiotic stresses such as zinc deficiency, a widespread micronutrient disorder affecting many soils worldwide. Zn deficiency can cause symptoms of oxidative stress in plants and increased ascorbate levels were suggested to enhance tolerance to zinc deficiency in rice. Ascorbate is an efficient antioxidant in plants and part of the defense system against oxidative stress. In this context, the ascorbate metabolism was investigated on a transcriptional and metabolomic level in two rice genotypes contrasting in field tolerance to zinc deficiency, RIL46 (tolerant) and IR74 (intolerant). The ability to maintain a higher ascorbate level caused by higher substrate availability and increased expression of some genes of the biosynthesis pathway was related to tolerance to zinc deficiency in RIL46. In contrast, recycling or catabolism of oxidized ascorbate was not a limiting factor under zinc deficiency in both genotypes. The process of oxidative stress formation was further investigated in different rice genotypes. One pair consisted of the above mentioned genotypes RIL46 and IR74 and another pair was represented by a TOS17 mutant line with a 20-30 % lower ascorbate level and its wild-type Nipponbare. Ascorbate protected plants efficiently from redox imbalances caused by zinc deficiency in shoots and roots. Moreover, visualization of hydrogen peroxide with different staining methods revealed that oxidative stress occurs even before the emergence of visible leaf symptoms. Additionally, T-DNA mutant lines with different ascorbate levels due to a knock-out of genes involved in the predominant D-mannose/L-galactose biosynthetic pathway were analyzed. A moderate decrease of ascorbate levels already caused increased sensitivity against abiotic stresses such as zinc deficiency and ozone stress, but not against iron toxicity. Plants with very low ascorbate levels showed severe growth defects and stress symptoms even under optimal conditions. Different ascorbate levels affected photosynthetic parameters, growth, developmental processes and yield parameters. Moreover, a knock-out in a gene taking part in a putative alternative biosynthetic pathway proceeding via *myo*-inositol had no impact on the ascorbate level. Thus it was suggested that the D-mannose/L-galactose biosynthetic pathway is the predominant one and that the alternative *myo*-inositol pathway presumably does not contribute to ascorbate biosynthesis in rice. In conclusion, ascorbate served as an efficient tolerance factor against abiotic stresses and enhanced plant fitness even under non-stressed conditions.

Zusammenfassung

Reis gehört zu den weltweit wichtigsten Grundnahrungsmitteln und aufgrund der wachsenden Weltbevölkerung wird der Bedarf an Nahrungsmitteln in den nächsten Jahren weiter steigen. Ertragsverluste können durch abiotische Stresse wie Zinkmangel verursacht werden und bedrohen die Ernährungssicherung. Zinkmangel ist ein in vielen Böden weltweit vorhandenes Problem und kann oxidativen Stress in Pflanzen auslösen. In diesem Zusammenhang wurden erhöhte Ascorbatkonzentrationen in Reis in Verbindung mit verbesserter Toleranz gegenüber Zinkmangel gebracht. Ascorbat ist ein effektives Antioxidans in Pflanzen und Teil des Abwehrsystems gegen oxidativen Stress. In diesem Kontext wurde der Ascorbat Metabolismus auf transkriptorischer und metabolischer Ebene in zwei Reis Genotypen untersucht, die unterschiedliche Toleranz gegenüber Zinkmangel zeigten, RIL 46 (tolerant) und IR74 (intolerant). Ein höherer Ascorbatgehalt, verursacht durch höhere Substrat Verfügbarkeit und stärkere Expression von Biosynthese Genen, führte zu Toleranz gegenüber Zinkmangel in RIL46. Im Gegensatz dazu waren die Regeneration und der Abbau von oxidiertem Ascorbat unter Zinkmangel in beiden Genotypen nicht eingeschränkt. Die Entstehung von oxidativem Stress wurde in verschiedenen Genotypen näher untersucht. Ein Paar bestand aus RIL46 und IR74, während ein weiteres Paar aus einer TOS17 Mutationslinie mit einem 20-30 % niedrigerem Ascorbatgehalt und ihrem Wildtyp Nipponbare bestand. Ascorbat schützte die Pflanzen erfolgreich vor einem durch Zinkmangel verursachten Redox Ungleichgewicht in Spross und Wurzeln. Durch die Visualisierung von Wasserstoffperoxid durch verschiedene Färbemethoden konnte gezeigt werden, dass oxidativer Stress schon vor sichtbaren Blattsymptomen auftrat. Zusätzlich wurden T-DNA Insertionslinien mit unterschiedlichem Ascorbatgehalt analysiert, die durch einen Knock-Out von verschiedenen Genen des vorherrschenden D-Mannose/L-Galactose Biosyntheseweges verursacht wurden. Ein nur mäßig verringerter Ascorbatgehalt verursachte erhöhte Sensitivität gegenüber abiotischen Stressen wie Zinkmangel und Ozonstress, jedoch nicht gegenüber Eisentoxizität. Pflanzen mit einem sehr niedrigen Ascorbatgehalt zeigten ein gestörtes Wachstum und sogar unter optimalen Bedingungen Stresssymptome. Verschiedene Ascorbatkonzentrationen beeinflussten die Photosynthese, das Wachstum, Entwicklungsprozesse und Erträge. Eine Mutationslinie für ein Gen des alternativen Biosyntheseweges via *Myo*-Inositol zeigte keine Veränderung im Ascorbatgehalt, woraus geschlossen wurde, dass dieser Weg vermutlich nicht zur Biosynthese beiträgt. Zusammengefasst lässt sich sagen, dass Ascorbat einen effektiven Toleranzfaktor gegen abiotische Stresse darstellt und die Pflanzenfitness auch unter nicht-gestressten Bedingungen verbessern kann.

Background

1.1 Rice as a model plant

Only 15 plant species are accounting for 90 % of the world`s food energy intake. The largest part derives from only three of them, rice (*Oryza sativa* L.*)*, maize (*Zea mays*) and wheat (*Triticum aestivum*), representing the staple food for over 4000 million people. Among these, rice can be regarded as the most important crop for human nutrition (Food and Agriculture Organization of the United Nations, FAO, www.fao.org). According to the FAO`s rice fact sheet of 2004, rice provides 20 % of the world`s dietary energy supply, while wheat provides 19 % and maize only 5 %. About 90 % of rice is grown in Asia and worldwide, more than 3.5 billion people depend on rice for more than 20 % of their daily calorie intake (International Rice Research Institute, IRRI, www.irri.org) (Fig. 1). The global 2013/2014 rice production is estimated to be around 750 million tonnes (495 million tonnes on a milled basis) (FAO Rice Market Monitor, April, 2014; United States Department of Agriculture, USDA, http://www.ers.usda.gov) (Fig. 2). However, an increase of 70 %-100 % of food production is required by the year 2050 (Godfray et al., 2010) and one strategy is to increase yields of important crops like rice. It has been estimated that the average irrigated rice yields achieved in South-East Asia are only 60 % of the potential maximum climate-adjusted yields (Cassman, 1999). While the global population is still growing, land and water resources providing the basis of sustainable rice production are declining. Important reasons for reduced yields of rice are environmental stresses, among which biotic and abiotic stress factors can be distinguished. Biotic stresses are caused by living organisms, such as bacteria, viruses, insects or parasites. Abiotic stresses include all non-living factors like extreme temperatures, droughts, salt stress or nutrient deficiencies or toxicities. One approach to overcome these limitations is the development of rice varieties tolerant to these stresses. Therefore a broad understanding of the causes and biochemical and genetic factors associated with tolerance mechanisms is essential. In our research, we focused on the impact of abiotic stresses and the reasons for tolerance in rice.

Rice is a monocotyledonous angiosperm and belongs to the genus *Oryza*, which contains about 20 other species. Two species are used as cultivated rice and are important for human nutrition, *Oryza sativa*, grown worldwide but mostly in South-East Asia and O*ryza glaberrima*, cultivated in West Africa. There are two major subspecies of *O. sativa*, which are *indica*, typically grown in lowlands of tropical Asia and *japonica*, grown in temperate East Asia and uplands of South-East Asia (Garris et al., 2005). Presumably, these two subspecies originated from separate domestication events (Cheng et al., 2003; Doi et al., 2002).

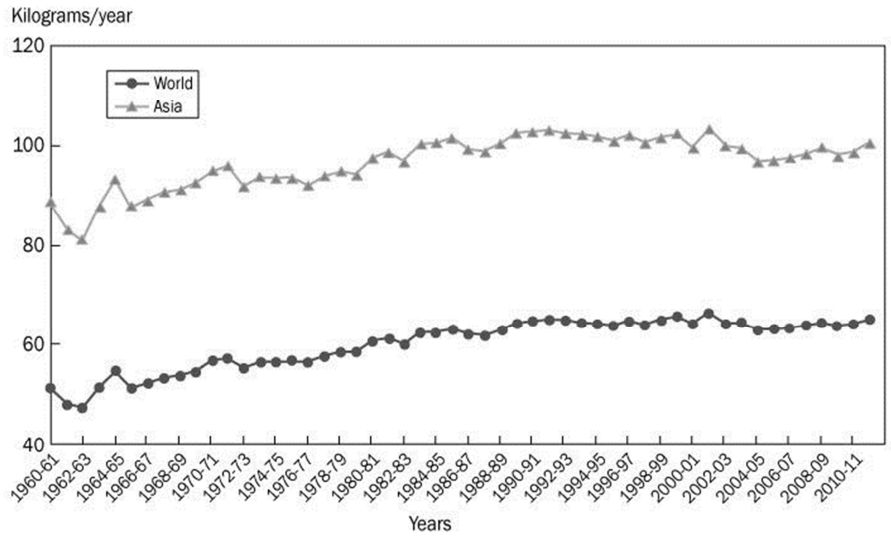
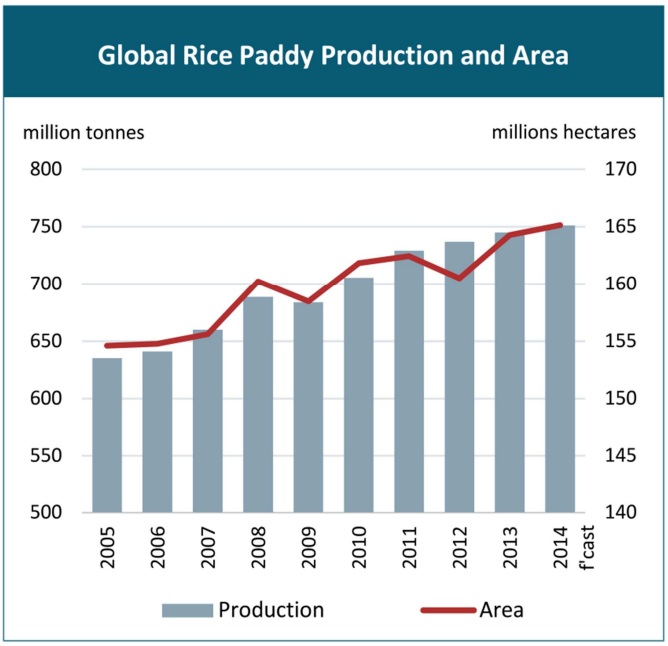


Fig. 1: Global and Asian per capita rice consumption. Data source: PSD online database (USDA) and FAOSTAT population database (FAO).

A great genetic biodiversity exists among different rice varieties, offering good opportunities for the development of new rice cultivars with improved performance under unfavorable conditions and enhanced nutritional value. The fact that rice was the first crop with a fully sequenced genome is an additional advantage facilitating the work with rice as a model plant (Goff et al., 2002; Project I.R.G.S., 2005). Rice has a diploid genome of relatively small size (0.4 Gb) compared to the hexaploid wheat genome (around 17 Gb) or the diploid maize genome (around 2.5 Gb), accelerating genomic research to a great extend (Eckardt, 2008). More than 230,000 germplasm accessions of rice exists in genebanks worldwide and 2012, the “3,000 (3K) Rice Genomes Project” started with the aim to sequence a broad spectrum of rice cultivars (Li et al., 2014). The sequence dataset for 3000 rice genomes (GigaScience

journal database, GigaDB, Sneddon et al., 2012) will help to elucidate genetic variation and provides a good basis for improved breeding applications.



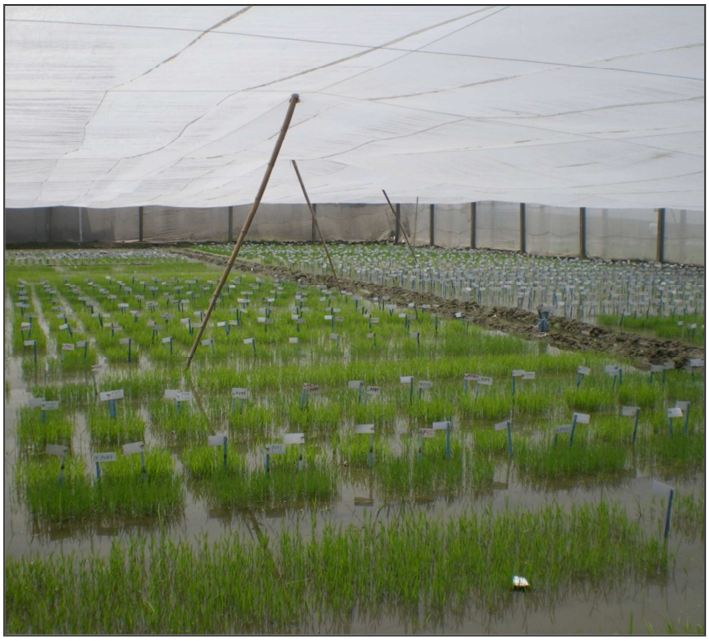


Fig 2: Global rice production and area (left), adapted from FAO Rice Market Monitor, April, 2014 and a rice paddy field at the research station of Yangzhou University, China (right).

1.2 Abiotic stress

Abiotic stresses like drought, salinity or nutrient toxicities or deficiencies are major reasons for yield losses (Dobermann and Fairhurst, 2000; Farooq et al., 2009; Marschner and Marschner, 2011; Shani and Dudley, 2001). Thus, the understanding of plant responses to abiotic stresses is of great importance for the development of tolerant species either through genetic engineering or conventional breeding methods. Plant abiotic stress resistance can be enhanced by different mechanisms like the production of osmolytes, regulation of ion- and water transport or the induction of reactive oxygen species (ROS) scavenging enzymes or molecules (Vinocur and Altman, 2005).

Zinc deficiency

A common nutrient deficiency causing severe yield losses is zinc (Zn) deficiency (Cakmak, 2000) (Fig. 3). Zn is one of the most abundant trace elements in organisms, even though it is only required in small amounts and the only element present in all six enzyme classes (Broadley et al., 2007). In solutions, Zn exists as Zn^{2+} and is redox stable. The primary ligand-binding sites for Zn^{2+} are structural sites to ensure correct protein folding and catalytic or cocatalytic sites to ensure the function of enzymes such as Cu/Zn superoxide dismutase (SOD) or purple acid phosphatases (Broadley et al., 2007). Mean Zn concentrations in soils worldwide range from 10 to 300 mg kg^{-1}, with a tendency of lower Zn amounts in sandy soils and higher concentrations in clay soils (Alloway, 2008). The main input of Zn into soils derives from weathering of parent rocks, while atmospheric and biotic processes can additionally contribute to the overall amount of Zn (Friedland and Shaw, 1990).

Fig. 3: A rice field at a research station at the International Rice Research Insitute (IRRI, Philippines) (left) with zinc deficient plots on the left and control plots on the right and an example of a single rice plant suffering from zinc deficiency (right) (pictures taken by Yoshiaki Ueda at the International Rice Research Insitute (IRRI), Philippines).

In soils, Zn occurs as water-soluble, adsorbed or exchangeable Zn in the colloidal fraction or as insoluble Zn in complexes or minerals. To which extent Zn is available for plants is mainly determined by the soil pH, since the amount of soluble Zn^{2+} increases with low pH, however, the largest proportion of soil Zn is insoluble (> 90 %) (Barber, 1995; Broadley et al., 2007).

Thus, symptoms of Zn deficiency are rather caused by low availability of Zn to plants than by the total amount of Zn in the soil.

Zn deficiency occurs mainly in South-East Asia, Australia, Turkey and Africa (Hacisalihoglu and Kochian, 2003) (Fig. 4) and is now considered to be one of the most widespread factors constricting rice production. It is estimated that around 50 % of all lowland rice soils are Zn deficient (Cakmak, 2008; White and Zasoski, 1999). One of the first symptoms of Zn deficiency appears around two to three weeks after transplanting as brown spots, termed as leaf bronzing that can cover the leaves entirely (Fig. 3). Moreover, plants suffer from growth delay and reduced yield (Neue and Lantin, 1994; Wissuwa et al., 2006). A major part of the produced rice crop is cultivated under flooded conditions resulting in increased phosphorous and bicarbonate concentrations in the soil, hindering the efficient uptake of Zn (Sharma et al., 2013). Other soil factors that have been associated with Zn deficiency are a high pH, low redox potential, high organic matter and low available Zn content (Neue and Lantin, 1994). The reason why Zn deficiency leads to oxidative stress in plants is not fully understood yet (possible reasons will be discussed in more detail in Chapter 2). Several root and shoot based mechanisms to increase tolerance to Zn deficiency have been proposed. The active release of Zn-mobilizing compounds like phytosiderophores and organic acids to increase Zn availability is considered to be the most important root-based mechanisms (Hoffland et al., 2006; Rose et al., 2011). Enhanced detoxification of ROS resulting from oxidative stress in the shoot ("shoot tolerance") is presumably an additional tolerance factor (Frei et al., 2010). The connection between increased oxidative stress resistance and an activation of protective oxidative stress genes was confirmed in a recent study with *Brachypodium distachyon.* An overexpression of a transcription factor (basic leucine zipper transcription factor, BdbZIP10) induced under Zn deficient conditions resulted in increased cell viability under oxidative stress, most likely through enhanced reduction of ROS (Glover-Cutter et al., 2014). However, further research is warranted to determine the factors causing oxidative stress formation as a result of Zn deficiency in rice and to investigate different tolerance mechanisms.

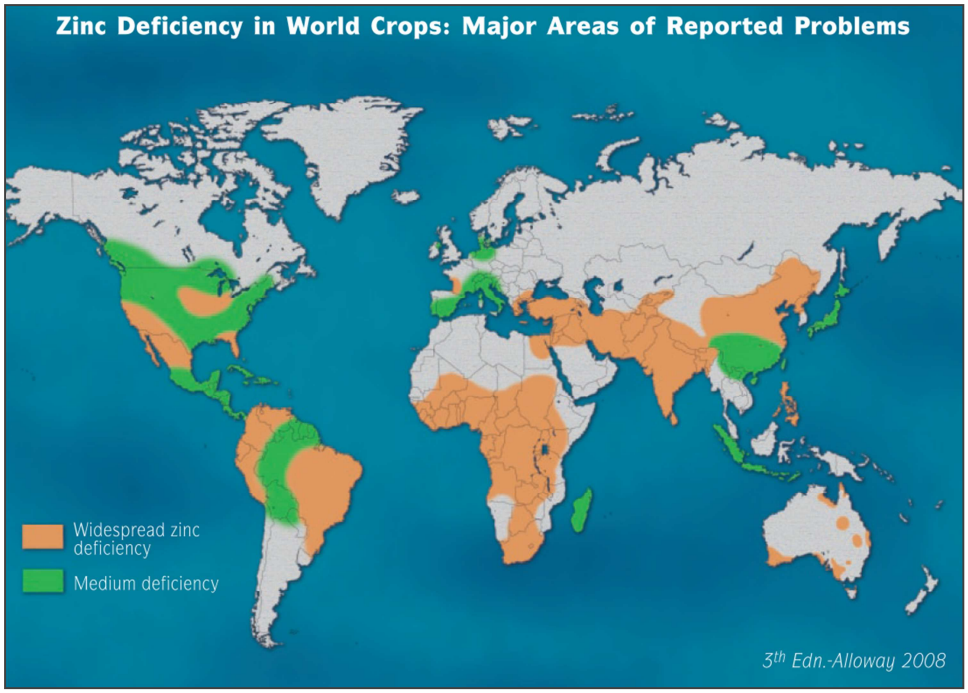


Fig. 4: Distribution of zinc deficiency in the world with severe zinc deficient areas marked in red and moderate affected areas marked in green. Adapted from Alloway (2008).

Ozone stress

Besides a growing world population, environmental factors like climate change and air pollution are serious threats for global food security. One of the most widespread air pollutants is ozone. Simulations predict that tropospheric ozone concentration will increase by 20-25 % by the year 2050 and the main reasons are the rising emission of gases such as nitrous oxides (Meehl et al., 2007). The predictions are even higher for tropical and subtropical countries in South-East Asia, India or Central America (20-80 % increase) (Grenfell et al., 2003). Ozone stress affects rice yield through an impaired photosynthesis, shoot growth, grain number and grain mass (Ainsworth, 2008). Compared to Zn deficiency, it is well known how ozone produces oxidative stress in the plant. Ozone enters the leaves through the stomata and is degraded to ROS. Due to increased ROS levels, photosynthetic capacity is decreased by an impairment of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) activity and stomatal function. Furthermore, reproduction can be affected as well as phloem loading and assimilate partitioning to roots and grain (Fuhrer, 2009).

Iron toxicity

Another factor concerning especially rice production is iron toxicity, since it only occurs in flooded soils (Becker and Asch, 2005). Together with Zn deficiency, iron toxicity is one of the most common nutrient disorders for lowland rice (Neue et al., 1998). An excess amount of reduced iron is taken up by the roots from the soil solution and translocated to the shoot. Like Zn deficiency and ozone stress, excess uptake of iron causes oxidative stress in the plant. Strategies to avoid damage of the plant caused by iron toxicity are I) exclusion: damage of the shoot by ferrous iron (Fe^{2+}) is avoided through rhizospheric oxidation or ion selectivity at the root level. II) Inclusion and avoidance: The avoidance of damage is conferred by internal compartmentation of ferrous iron in older leaves or apoplastic immobilization. III) Inclusion and tolerance: Plants develop strategies to cope with a higher ferrous iron concentration in the shoot, e.g. enhanced ROS detoxification (Asch et al., 2005).

1.3 Oxidative stress

All of the above mentioned stress factors have in common that they produce symptoms of oxidative stress in the plant, caused by an imbalance of production and removal of ROS. The generation of ROS and detoxification systems co-evolved along with aerobic processes like photosynthesis and respiration. ROS are defined as partially reduced forms of atmospheric oxygen (O_2) and are produced by an excitation of atmospheric oxygen to its singlet form (O_2^1) or by a subsequent electron transfer resulting in the formation of the superoxide radical ($O_2\cdot^-$), the peroxide ion (O_2^{2-}) or hydrogen peroxide (H_2O_2). Furthermore, the Haber-Weiss-reaction, which is part of the Fenton-reaction can form the hydroxyl radical (OH·) in the presence of transition metal ions (Gill and Tuteja, 2010; Winterbourn, 1995) (Fig. 5). These ROS have been considered as unavoidable byproducts of the aerobic metabolism for a long time, leading to damage of proteins, lipids and DNA. However, in the recent years it became apparent, that they possess different important functions like signaling and regulation of processes such as cell growth (D'Autréaux and Toledano, 2007; Gapper and Dolan, 2006). Contrary to previous assumptions, ROS are even actively produced in plants in processes such as programmed cell death or pathogen response. Major ROS production sites in the cells are e.g. chloroplasts, mitochondria and peroxisomes, due to aerobic processes like photosynthesis or respiration. Especially photosynthesis accounts for large amounts of ROS since oxygen is photoreduced

to H_2O_2 in photosystem (PS) I and triplet oxygen is excited to its singlet state in PSII (Asada, 2006). However, there are several other cellular sources of ROS such as NAD(P)H-oxidases, triggering the oxidative burst, a rapid production of huge amounts of ROS. The oxidative burst is part of a hypersensitive response to cause defined damage of cells in the site of pathogen attack (Lamb and Dixon, 1997). Amine oxidases and peroxidases are additionally considered to produce ROS. These production processes have to be controlled tightly to avoid damage such as lipid peroxidation, protein oxidation and enzyme inhibition (Mittler, 2002). However, different abiotic stresses can lead to increased production of ROS and can disturb the redox homeostasis of cells. These include drought and salt stress, heavy metals, heat shock, high light, nutrient toxicities or deficiencies, air pollutants, UV radiation and mechanical stresses (Dat et al., 2000; Mittler 2002). These ROS constitute a threat for the cells but are additionally considered to be part of a signaling pathway to trigger stress responses (Knight and Knight, 2001). Thus, it was necessary for plants to develop an antioxidant system to keep the production and removal of ROS in balance.

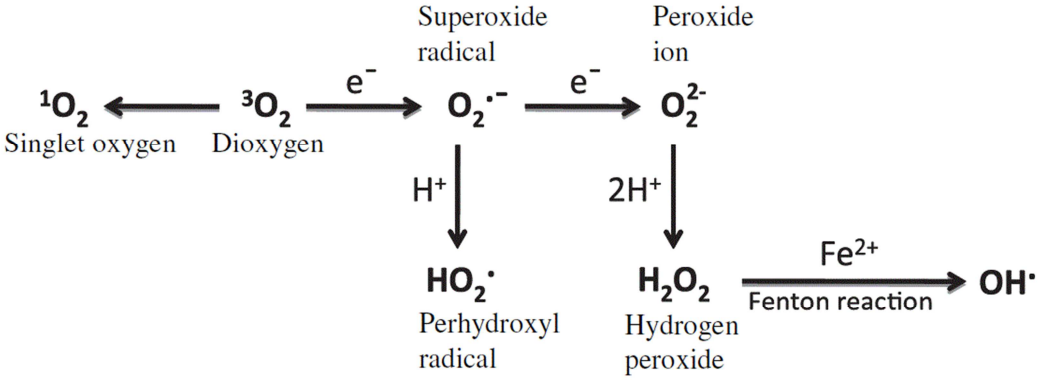


Fig. 5: Generation of reactive oxygen species by energy transfer. Adapted from Gill et al. (2010).

Detoxification of reactive oxygen species

Especially in abiotic or biotic stress situations, plants have to deal with an excess production of ROS, which is conducted by different antioxidants. An antioxidant is capable of reducing harmful molecules without being toxic in its oxidized form. The detoxification of ROS can be divided in non-enzymatic and enzymatic scavenging mechanisms (Apel and Hirt, 2004). The most efficient non-enzymatic ROS scavengers are ascorbic acid (AsA) and glutathione. AsA

can reduce H_2O_2 to water by donating two electrons because of its ability to delocalize electrons around its 5-carbon ring (Fig. 6). It can interact with hydroxyl radicals, singlet oxygen, superoxide, but also glutathione and tocopherol radicals (Noctor and Foyer, 1998). Recycling of reduced AsA occurs enzymatically during the ascorbate-glutathione cycle; however, if the recycling capacity is insufficient, AsA is degraded to different products (the AsA metabolism is explained in more detail in Chapter 1). Glutathione can reduce ROS directly or act as an electron donor in the recycling step for AsA (Fig. 6).

Additionally, tocopherols, flavonoids and carotenoids can function as non-enzymatic ROS scavengers. Tocopherols are considered as fat-soluble antioxidants in biomembranes with potential ROS and lipid radical scavenging functions. AsA is required as a reductant to regenerate α-tocopherol after detoxification of ROS (Niki, 1987). The reduction potential of flavonoid radicals is responsible for their activity as ROS scavenging molecules. Flavonoids are among the most bioactive plant secondary metabolites and posses many other functions like pigmentation of fruit, flowers and seeds, protection against UV-light or as signaling molecules in plant-microbe interactions (Gill and Tuteja, 2010). Carotenoids are especially important in dissipating excess energy during photosynthesis and scavenging photoproduced ROS. Moreover they possess a light-harvesting function and can transfer light at wavelengths between 400-550 nm to chlorophyll molecules (Siefermann-Harms, 1987).

One of the most effective enzymatic intracellular antioxidants is SOD, a metalloenzyme present in all living aerobic organisms (Bowler et al., 1994). SOD constitutes the first step in ROS detoxification since it catalyzes the dismutation of $O_2^{\bullet-}$ by reducing one molecule of $O_2^{\bullet-}$ to H_2O_2 and oxidizing another to one molecule of O_2. Three types of SODs are known, differing in their metal cofactors: the copper/zinc SOD (Cu/Zn SOD), manganese SOD (Mn-SOD) and iron-SOD (Fe-SOD) (Gill and Tuteja, 2010). In a next step, H_2O_2 can dismutate to H_2O and O_2 catalyzed by catalase (CAT), a haem-based antioxidant enzyme. In contrast to other antioxidant enzymes, like ascorbate peroxidase (APX), it does not require a reductant, since it only catalyzes a dismutation (Mhamdi et al., 2010). As a part of the ascorbate-glutathione cycle APX catalyzes the reduction of H_2O_2 to H_2O with AsA as an electron donor. The first oxidation product monodehydroascorbate (MDHA) can delocalize electrons around a central carbon ring and its three carbonylgroups, resulting in high stability for a radical. Another enzyme taking part in the ascorbate-glutathione cycle is glutathione reductase (GR), a flavoprotein oxidoreductase, catalyzing the NADPH dependent reduction of oxidized

glutathione. Additionally, guaiacol peroxidase is able to detoxify H_2O_2 by decomposing indole-3-acetic acid (Gill and Tuteja, 2010). The main substrates for guaiacol peroxidase are aromatic substances like guaiacol and pyragallol, but it is also capable of oxidizing AsA at a very low rate.

1.4 Ascorbate

A previous study revealed that tolerance to Zn deficiency in a Zn efficient genotype derived from a rice mapping population was accompanied by a higher AsA level compared to its intolerant parent. Therefore this study focuses on investigating the impact of AsA on tolerance to different stresses and the reasons for the ability of some genotypes to maintain higher AsA levels than others. AsA is an ubiquitous molecule especially present in the leaves and fruits of higher plant, which provides the major source of vitamin c for humans, who are not able to synthesize it. Concentrations differ among species and are dependent on tissue type, light intensity and plant age (Gest et al., 2013). AsA is an abundant antioxidant in higher plants and concentrations usually differ between 2-20 μmol g^{-1} FW, however, they can be several fold higher e.g. in alpine plants, presumably because of the high-light intensities and low temperatures (Smirnoff 2000b; Smirnoff et al., 2001). During the evolution of plants, AsA concentrations constantly increased, highlighting its important role as an effective antioxidant. AsA is a relatively small molecule which is synthesized from glucose as the main precursor, however, only a small amount of the total glucose pool is required for its biosynthesis. Additionally, AsA biosynthesis does not consume much energy, thus, it is possible to synthesize it in large amounts (Gest et al., 2013). Briefly, AsA is synthesized via a pathway involving the conversion of hexose sugars to D-mannose and L-galactose, catalyzed by several enzymatic steps (Smirnoff et al., 2001). However, the first part of the biosynthetic pathway is not only committed to AsA biosynthesis but additionally delivers precursors for cell wall components (Smirnoff, 2000a). Although the D-mannose/L-galactose pathway is considered as the predominant pathway, it is well established that alternative pathways via uronic acid intermediates (Agius et al., 2003; Davey et al., 1999) and GDP-L-gulose (Wolucka and Van Montagu, 2003) exist. The possible conversion of *myo*-inositol into AsA was investigated very early (Loewus et al., 1962), but it is still discussed whether the *myo*-inositol pathway really contributes to AsA biosynthesis. The AsA metabolism will be

explained in more detail in Chapter 1.

Concerning the intracellular distribution, chloroplasts, the nucleus and peroxisomes contain relatively high concentrations of AsA (Zechmann et al., 2011). The symplast contains the majority of AsA, but AsA is present in the apoplast as well, maintained primarily in its oxidized form by the enzyme ascorbate oxidase (AO). Since AO oxidizes AsA and therefore hinders its function as an antioxidant in its active form, its biological function is still poorly understood. However, several experiments showed that faster cell expansion and elongation correlated with higher AO activity (Pignocchi et al., 2003). This might be explained by the fact that cell wall expansion is induced by ROS (Joo et al., 2001) and reduced AsA activity can therefore promote cell growth.

Apart from its role as an antioxidant, AsA is in involved in a number of different cellular reactions, like photoprotection, plant development and cell expansion. A reason for the complex roles of AsA might be its moderate redox potential (Noctor, 2006). Thus, AsA is able to take part in a lot of reactions in the cellular metabolism without being too reactive.

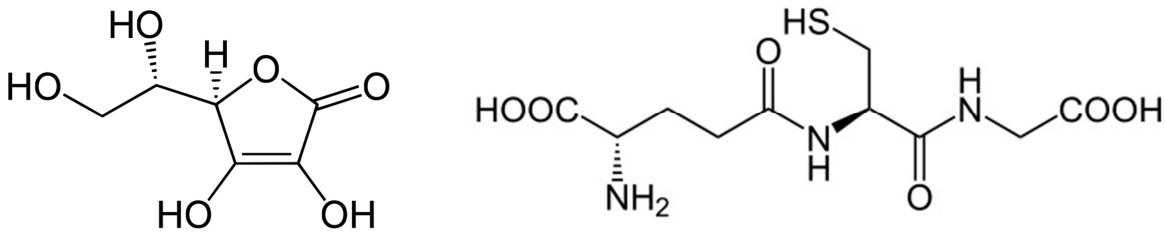


Fig. 6: Chemical structure of ascorbate (left) and glutathione (right).

1.5 Aims of research

I. Previous results suggested an involvement of AsA as an important antioxidant in tolerance to Zn deficiency in two rice genotypes contrasting in Zn deficiency (Frei et al., 2010; Wissuwa et al., 2006). The tolerant genotype (RIL46) was able to maintain a higher AsA level under Zn deficiency than the intolerant one (IR74). In a first approach these two genotypes were used to analyze differences in AsA metabolism in more detail by metabolomic and transcript profiling. We established two hypotheses to explain the ability of the tolerant genotype to maintain a higher AsA level:

1. AsA biosynthesis is impaired in the intolerant IR74 but not in the tolerant one.
2. Insufficient recycling of reduced AsA leads to degradation in the intolerant genotype.

II. It is well established that Zn deficiency leads to an excess formation of ROS and oxidative stress, but the reasons remain unclear. Zn is involved in numerous processes in the cell including gene expression, protein metabolism and as a cofactor for many enzymes including those that are involved in redox processes like the Cu/Zn SOD (Broadley et al., 2007). Using the above mentioned pair RIL46/IR74 and a TOS17 mutant line with a 20-30 % lower AsA level than its wild-type, we tested the following hypotheses:

1. Oxidative stress is caused by Zn deficiency in shoots and roots and precedes visible symptoms like leaf bronzing.
2. AsA acts as a tolerance factor in both shoots and roots against oxidative stress by maintaining redox homeostasis.

III. To confirm the results concerning the role of AsA in oxidative stress protection T-DNA insertional lines and the above mentioned TOS17 mutant for different AsA biosynthesis genes were used. It was found that a gene taking part in a possible alternative biosynthetic pathway via *myo*-inositol was upregulated in the tolerant line RIL46. Thus, a mutant with a knock-out in a gene coding for *myo*-inositol oxygenase (MIOX) was used. It was tested if AsA can protect plants against different abiotic stresses such as ozone stress and iron toxicity. Since AsA has several roles in the plants metabolism, a long-term experiment with the mutants was conducted to elucidate the impact of different AsA levels on plant growth, flowering and yield. Moreover, its impact on photosynthesis was analyzed.

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Chapter 1: Ascorbate metabolism in rice genotypes differing in zinc efficiency

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1.1 Abstract

Effects of zinc (Zn) deficiency on shoot metabolites were investigated in contrasting rice (*Oryza sativa* L.) genotypes with special focus on ascorbic acid (AsA) biosynthesis, recycling, and catabolism. The genotypes IR74 (sensitive) and RIL46 (tolerant) were subjected to –Zn and control treatments for three weeks, and samples were taken at three different stages representing the pre-stress phase, emergence of visible stress, and severe visible stress. The emergence of visible symptoms was paralleled by an increase in lipid peroxidation and a decrease in AsA concentration in the sensitive but not in the tolerant genotype. The tolerant RIL46 showed enhanced transcript levels of several genes involved in the mannose/L-galactose pathway to AsA biosynthesis, and significant up-regulation of a gene involved in the putative alternative *myo*-inositol pathway under low Zn stress. The level of most AsA precursors was negatively affected by Zn deficiency, but RIL46 had a constitutively higher level of non-phosphorylated precursors. Products of AsA catabolism such as oxalate and threonate did not accumulate in either genotype, suggesting that AsA degradation did not contribute to the stress induced decline of the AsA pool in IR74. Further factors possibly contributing to tolerance in RIL46 included an almost 5-fold higher proline level under –Zn stress and significantly higher trehalose content. The implications of these compounds in AsA metabolism and Zn efficiency thus deserve further attention.

Keywords: Ascorbate biosynthesis, Micronutrients, Proline, Reactive oxygen species**,** Zinc deficiency

1.2 Introduction

Zinc (Zn) deficiency is one of the most abundant abiotic stress factors worldwide and affects growth and yield of rice (*Oryza sativa* L.) and other crops on approximately 35 million hectares of agricultural land (Alloway 2004). Zn is essential for plants and has numerous functions in the cell, such as involvement in protein folding, or as a catalytic or co-catalytic factor of thousands of proteins (Broadley et al. 2007). Similar to other abiotic stress factors, Zn deficiency can induce an imbalance in the production and removal of reactive oxygen species (ROS), thereby causing oxidative stress in plants (Cakmak and Marschner 1988). Although ROS are constantly produced in plant cells and have important signaling functions, their excessive production causes damage of lipids, proteins and DNA (Apel and Hirt 2004).

The breeding of rice varieties tolerant to Zn deficiency (also termed as 'zinc efficient') is of great importance to global agriculture. Genetic evidence shows that Zn efficiency is caused by multiple tolerance factors in rice, where several quantitative trait loci (QTL) with complex interactions influence plant mortality and leaf bronzing of plants grown on low Zn soil (Wissuwa et al. 2006). Also, the scientific literature suggests that Zn efficiency relies on at least two components: (i) the ability to acquire sufficient Zn despite low availability in the soil, and (ii) efficient utilization of Zn within the plant, also termed as "shoot tolerance (Hacisalihoglu and Kochian 2003). Both tolerance factors have been investigated in previous studies using the Zn efficient recombinant inbred line RIL46 selected from a rice mapping population, which showed enhanced growth, lower mortality and fewer stress symptoms when grown in low Zn soil compared to its intolerant parent IR74 (Wissuwa et al. 2006; Frei et al. 2010b; Rose et al. 2012). For instance, it was demonstrated that rhizosphere processes contribute to Zn efficiency, especially the ability to take up Zn chelated by phytosiderophores or organic acids (Hoffland et al. 2006; Wissuwa et al. 2006; Arnold et al. 2010). However, the tolerant line RIL46 showed an even lower Zn concentration in the shoot than its intolerant parent IR74. This observation is in agreement with findings from other crops, where Zn efficient and inefficient genotypes often do not differ in Zn concentration (Hacisalihoglu and Kochian 2003), and highlights the importance of an efficient biochemical utilization of Zn within the shoot. Indeed it was reported that RIL46 was able to protect cells from excess ROS more efficiently than IR74 (Frei et al. 2010b; Rose et al. 2011). Screening of the activity and

shoot concentrations of enzymatic and non-enzymatic antioxidants led to the conclusion that tolerance to Zn deficiency was associated with an enhanced ascorbate (AsA) level (Frei et al. 2010b). Unlike most antioxidant enzymes (such as superoxide dismutase, peroxidases, etc.), the level of AsA decreased when plants encountered low Zn stress, but remained higher in the tolerant genotype RIL46.

AsA is the most abundant antioxidant in plants and its metabolism can be divided into three sections (Fig. 1): (i) biosynthesis, (ii) recycling and (iii) catabolism (or degradation):

(i) Biosynthesis: the predominant biosynthesis pathway of AsA starts from hexose sugars and proceeds via GDP-D-mannose and L-galactose (mannose/L-galactose pathway) (Wheeler et al. 1998; Smirnoff and Wheeler 2000; Conklin 2001), but evidence for other pathways via uronic acid intermediates exists (Agius et al. 2003; Wolucka and Van Montagu 2003) (Fig. 1). One alternative pathway involving the conversion of D-galacturonic acid to L-galactono-1,4-lactone was verified in strawberry fruit (Agius et al. 2003; Loewus and Kelly 1961) and *Arabidopsis thaliana* cell suspension culture (Davey et al. 1999), but not in rice (Jo and Hyun 2011). Another alternative pathway uses *myo*-inositol as a substrate (Lorence et al. 2004) and is mediated by the enzyme *myo*-inositol oxygenase (MIOX). The contribution of the *myo*-inositol pathway to AsA in plants is under debate, as contradictory results have been reported (Lorence et al. 2004; Endres and Tenhaken 2009).

(ii) Recycling: the enzyme ascorbate peroxidase (APX) uses two molecules of AsA to reduce H_2O_2 to water, leading to the generation of the unstable molecule monodehydroascorbate (MDHA), which disproportionates to dehydroascorbate (DHA) if not reduced back to AsA by the action of monodehydroascorbate reductase (MDHAR). DHA is recycled to AsA using reduced glutathione (GSH) as a reducing substrate, which is catalyzed by DHA reductase (DHAR) (Noctor and Foyer 1998; Smirnoff 1996).

(iii) Degradation: If DHA is not reduced quickly, it decomposes due to irreversible hydrolysis of its lactone ring, leading to the formation of diketogulonic acid (Green and Fry 2005). Additionally, DHA can be cleaved to form oxalate (C-1 and C-2) and L-threonate (C-3 to C-6), which is often oxidized to L-threarate (DeBolt et al. 2004). Green and Fry (2005) proposed that two interchangeable intermediates are formed (cyclic -2,3-O-oxalyl-L-threonate and cyclic -3,4-O-oxalyl-L-threonate) and subsequently, the latter is hydrolyzed to 4-O-

oxalyl-L-threonate. It was suggested that this pathway is at least partially catalyzed by yet uncharacterized enzymes.

As our previous results using the contrasting genotypes IR74 and RIL46 suggested that Zn efficiency was associated with the ability to maintain an enhanced AsA pool, the present study focused on genotypic differences in AsA metabolism by targeted metabolite and transcript profiling. More specifically we investigated two hypotheses (i) Zn deficiency inhibits AsA biosynthesis in the intolerant genotype but not in the tolerant one. To test this hypothesis we focused on transcriptional and metabolic regulation of the different biochemical routes affecting AsA levels. (ii) Alternatively, insufficient AsA recycling leads to degradation of AsA in the intolerant genotype, but not in the tolerant one. More specifically, excess production of ROS due to stress may exhaust the enzymatic AsA recycling capacity and consequently lead to irreversible degradation of AsA. Testing these hypotheses was part of a broader metabolomic approach, which included the amino acid and carbohydrate profile to identify further factors interacting with the AsA metabolism and contributing to shoot tolerance under Zn deficiency.

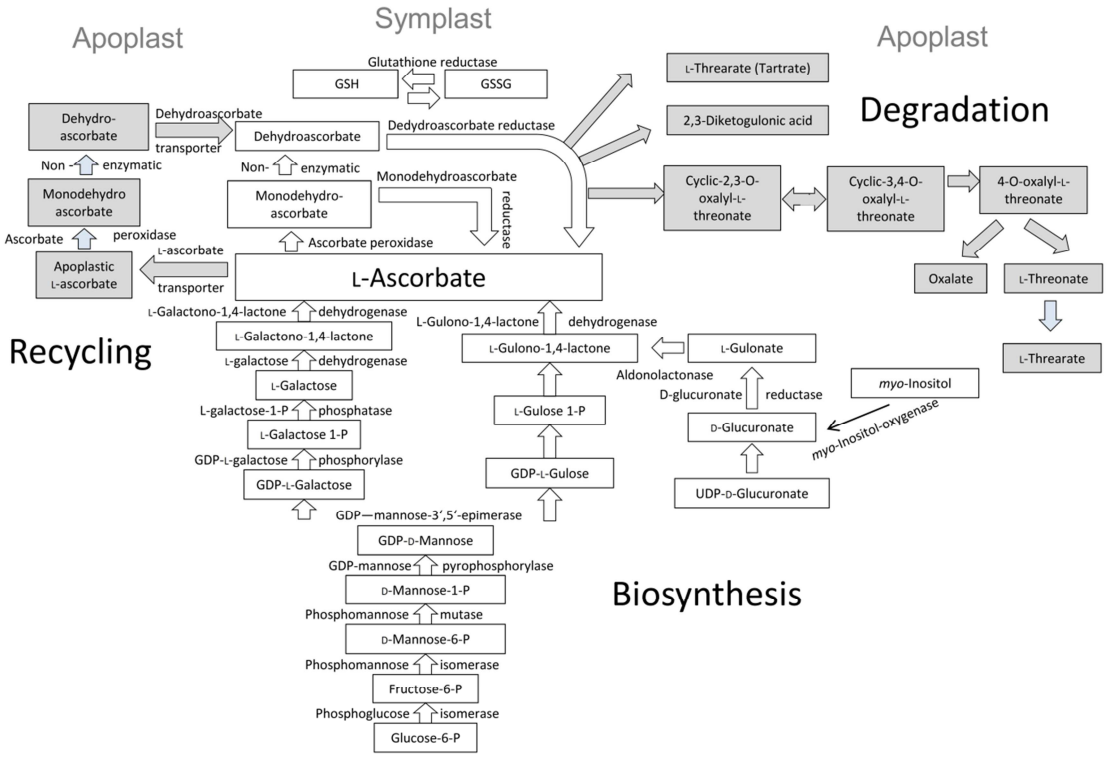

Fig. 1 Proposed scheme of the ascorbate metabolism in rice; boxes represent substrates (gray-shaded boxes indicate apoplastic steps, white indicates symplastic steps), arrows represent enzymes.

1.3 Materials and methods

Plant culture

The experiment was conducted using a Zn efficient recombinant inbred rice (*Oryza sativa* L.) line RIL46, which carries positive alleles of QTLs for tolerance to low Zn (Wissuwa et al. 2006), and its Zn-inefficient parent IR74. Seeds were obtained from the Japan International Research Institute for Agricultural Sciences (JIRCAS, Tsukuba, Japan). They were germinated and placed on styrofoam sheets floating on 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$. After 10 days seedlings were transferred to 60 l hydroponic tanks containing half strength nutrient solution without Zn. After one week plants were transferred to full nutrient solution containing 1mM $NaHCO_3$ (Rose et al. 2011) without (–Zn) or with 1 µM Zn (+Zn). The composition of the nutrient solution was: NH_4NO_3 1.42 mM, NaH_2PO_4 0.32 mM, K_2O_4 0.51 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, $MnCl_2$ 9 µM, $(NH_4)_6$ x Mo_7O_{24} 0.07 µM, H_3BO_3 18.5 µM, $CuSO_4$ 0.16 µM, $FeCl_3$ 35.6 µM. The pH of the solution was adjusted to 5.5 twice weekly and nutrient solutions were completely replaced after 10 days. Plants were grown in a temperature-controlled glasshouse with minimum night/day temperatures of 22°C/28°C. Average relative humidity was 50 %. Plants were grown under supplementary artificial lighting to ensure a minimum photosynthetic photon flux density (PPFD) of 400 µmol m^{-2} s^{-1} from 7 am to 8 pm.

Samples of whole shoots were harvested on three different sampling days: firstly three days after the start of –Zn treatments; secondly when stress symptoms first emerged in the susceptible genotype after 16 days of –Zn treatment and finally when IR74 had severe stress after 24 days of –Zn treatment. A leaf bronzing score ranging from 0 (healthy leaf) to 10 (dead leaf) was assigned to the three youngest fully expanded leaves of each plant (Wissuwa et al. 2006).

Gene expression analyses

Samples (ten replicates per genotype/treatment combination per sampling day) were immediately frozen in liquid N_2 and stored at -80°C. RNA for quantitative RT-PCR was extracted from 4 replicate samples using the RNeasy Plant Minikit (Qiagen). DNA was removed from RNA samples by using an RNase-free DNase (Promega). RNA concentration

was determined using a Nanodrop2000C spectrometer (Thermo Scientific) and the integrity of RNA was checked by denaturing formaldehyde agarose gel electrophoresis. One µg of total RNA was reverse transcribed with the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany) and quantitative real time polymerase chain reaction (qPCR) was performed with 100 ng cDNA using the GoTaq® qPCR Master Mix (Promega) under the following conditions: An initial denaturation step (20 min, 95°C), followed by 45 cycles of denaturation (5 sec, 95°C) and annealing/extension (40 sec, 60°C). Gene specific primers (Supplementary Table S1) were used for analysis of the different isoforms of all annotated biosynthesis genes of the mannose/L-galactose pathway and the *myo*-inositol pathway. Expression data were quantified using the comparative $\delta\delta C_T$ method (Frei et al. 2010a) using the expression level of IR74 in the control on the first sampling day as calibrator and the gene Os05g0564200 (RAP-ID, annotated as U2 snRNP) as endogenous reference to normalize gene expression levels of the targeted genes. The endogenous reference gene was identified using the database "Plant reference gene server" (Patel and Jain 2011) (http://www.nipgr.res.in/PlantRGS/), as it showed more stable expression across genotypes under the experimental conditions as compared to commonly used references such as actin or 18S rRNA.

Biochemical analyses

Zn concentration was determined in dried leaf material by atomic absorption spectrometry (AAS).

To measure malondialdehyde (MDA) (Hodges et al. 1999), leaf material was ground in liquid nitrogen and extracted twice by adding 1 ml of 0.1 % TCA to 100 mg of plant material and centrifugation (14000 *g*, 4°C, 15 min). The combined supernatants were divided into two 750 µl samples and 750 µl of 0.01 % (v/v) 2,6-di-tert-butyl-4-methylphenol (BHT) in 20 % TCA (v/v) was added to one sample. Seven hundred and fifty µl of 0.01 % (v/v) BHT and 0.65 % 2-thiobarbituric acid (TBA) in 20 % TCA (v/v) were added to the other sample. Samples were incubated at 95°C for 30 min and the reaction was stopped on ice. Samples were centrifuged (8000g, 4°C, 10 minutes) and absorbance was read at 440, 532 and 600 nm.

Total and reduced AsA were measured immediately after plant harvest according to Ueda et al. (2013). Flash-frozen leaves were ground in liquid nitrogen and 1.5 ml of 6 % trichloroacetic acid (TCA) was added to approximately 80 mg of leaf material. Samples were

incubated in a chilled ultrasonic water bath for 15 min and centrifuged for 5 min at 4°C and 13000 *g*. Ten µl of the supernatant was used to determine total and reduced AsA. Ten µl of 75 mM phosphate-buffer was added to all samples, while 10 µl of 10 mM dithiothreitol (DTT) was added only to the total AsA samples and incubated for 15 min at room temperature. Then, 10µl of 0.5 % N-Ethylmaleimide (NEM) was added to the total AsA samples to remove excess DTT and incubated for at least 30 s. Twenty µl of water was added to the reduced AsA samples to account for the amount of DTT and NEM. Thereafter, 50 µl of 10 % TCA, 40 µl of 43 % H_3PO_4, 40 µl of 4 % α-α-bipyridil and 20 µl of 3 % $FeCl_3$ was added to all tubes. After incubation at 37°C for 1 h, 100 µl of each sample was transferred to a 96 well microplate and absorbance was read at 525 nm using a microplate reader (Powerwave XSII, BioTec). The amount of DHA was calculated as the difference between the total and reduced AsA concentration.

Metabolomic analyses

Amino acids and sugars were analyzed using either a reverse phase chromatography or an enzyme-coupled photometric assay according to (Ahkami et al. 2009). Samples were incubated for 60 min at 80°C in 80 % ethanol and centrifuged for 10 min at 18 000 *g* The supernatants were evaporated to dryness and samples re-suspended in milliQ water. Prior to HPLC analysis samples were derivatized using a fluorescing reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamat). Three mg of self-made AQC was dissolved in 1 ml acetonitrile and incubated for 10 min at 55°C. The reagent was stored at 4°C and used up to four weeks. For derivatization of primary and secondary amino acids, 0.16 ml of a buffer containing 0.2 M boric acid, pH 8.8 was used. An aliquot of 0.02 ml of each sample was added to the buffer, followed by 0.02 ml of prepared AQC reagent solution. The whole mixture was incubated for 10 min at 55°C and used for chromatographic separation.

The concentrations of amino acids were determined using a reversed phase Alliance HPLC system (Waters Company, Germany) connected to a fluorescence detector (300 nm excitation wavelength, and 400 nm emission wavelength). Chromatograms were recorded using the software program Empower 2.1. The gradient was accomplished with a buffer A containing 140 mM sodium acetate, pH 5.8 (Suprapur, Merck) and 7 mM triethanolamine (Sigma, Germany). Acetonitrile (Roti C Solv HPLC, Roth) and milliQ water were used as eluents B and C. To separate the amino acids a reversed phase column (AccQTag, 3.9 mm x 150 mm) was used, which consists of silica as matrix modified by an apolar C18 group. The column

was equilibrated with buffer A at a flow rate of 0.6 ml per minute and heated at 37°C during the whole measurement. The gradient was produced by the following concentration changes: Start with 100 % eluent A, 1 % at 0.5 min, 5 % at 27 min, 9 % at 28.5 min, 18 % at 44.5 min, 60 % at 47.5 min and 0 % at 50.5 min of eluent B. Reconditioning of the column was carried out for 10 min using buffer A.

To measure soluble sugars, the same extracts as described above for amino acid preparation were used. The measurement was carried out with a microplate reader (Synergy HT, Tecan Germany). Auxiliary enzymes glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, 1 U), hexokinase (1 U), phosphoglucose isomerase (0.3 U) and 1 µl of ß-fructosidase (10 mg/100 µl buffer without NAD and ATP) were successively added to a buffer containing 100 mM imidazol-HCl, pH 6.9, 5 mM $MgCl_2$, 2 mM NAD and 1 mM ATP to detect glucose, fructose and sucrose. The difference in absorbance was proportional to the concentration of the individual sugars.

Sugar alcohols were detected using an ion chromatography system (Dionex, Idstein, Germany) which consisted of a gradient pump (GP50), a degaser module, an autosampler (AS50) and a pulsed amperometric detector (EG50). Separation of the sugar alcohols were carried out using a CarboPack MA1 column (4 x 250 mm) connected to a guard column of the same material (4 x 10 mm) and an ATC-1 anion trap column which was placed between the eluents and separation columns to remove the anionic contaminants present in the eluents. A linear gradient was accomplished with purest water as buffer A (Millipore) and 620 mM sodium hydroxide as buffer B (Baker, 50 % solution). The column was equilibrated at a flow rate of 0.4 ml per minute. The duration of the run was 60 minutes. The calibration and quantitative calculation of sugar alcohols was carried out using the Chromeleon software 6.6 and authentic standards.

All other metabolites were measured using a Dionex ICS 5000 (Dionex) coupled to a mass spectrometer, Agilent 6490 triple Quad. The ICS system consisted of a pump (DC), an eluent generator (EG) and an autosampler (AS-AP) module. Separation of the metabolites was carried out using AS11-HC column (2 x 250 mm) connected to a guard column of the same material AG11-HC (2 x 50 mm) and an ATC anion trap column which was placed between the eluents and separation columns to remove the anionic contaminants present in the eluents. A gradient was accomplished with purest water (eluent A, Millipore 18.2 microOhm) and increasing concentration of potassium hydroxide from a concentrated EluGen Cartridge EGC-

KOH (Dionex). The column was equilibrated at a flow rate of 0.32 ml per minute with 4 % KOH. The gradient was set as follow: 4 % for 4 minutes, 4-10 min to 15 %, 10-14 min to 25 %, 14-24 min to 80 %, 24-30 min back to 4 % and 30-40 min equilibration at 4 % KOH. The duration of the run was 40 minutes. The calibration and quantitative calculation of organic acids was carried out using the Chromeleon software 7.1.

Detection of accurate masses for different compounds was carried out on an Agilent triple Quad MS 6490 equipped with an easy Jet Spray. Data were acquired and evaluated via Mass Hunter (version B.04.00). The MS was operated in the negative ion and multiple reactions monitoring (MRM) mode. Single analyte standards were infused at a flow rate of 0.4 ml/min for tuning compound dependent MS parameters. The major MS/MS fragment patterns of each analyte were determined. Declustering potential (DP) and collision energy (CE) of each transition was optimized. The optimized parameters were as following: nitrogen gas flow: 12 l per min, ion spray voltage: −3500V, the auxiliary gas temperature: 350°C. The curtain and collision gas was nitrogen generated from pressurized air in a nitrogen generator (SF4, Atlas Copco, Magdeburg, Germany). Dwell time for each transition was 20 ms.

Enzyme assays

DHAR activity was measured according to Hossain and Asada (1984). Approximately 100 mg of flash-frozen leaf material was crushed in liquid nitrogen and 1.5 ml extraction buffer containing 50 mM Tris-HCl, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM $MgCl_2$ was added. Samples were centrifuged at 13000 *g* for 5 min at 4°C and a reaction mix with 70 µl of 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.5, 10 µl of 5 mM dehydroascorbate (DHA), 10 µl of 50 mM reduced glutathione (GSH) was added to 10 µl of the enzyme extract. Absorbance was followed in a 96 well microplate at 265 nm for 3 min (ε= 14 mM^{-1} cm^{-1}). Non-enzymatic reduction of DHA was measured using a blank sample without enzyme, and the value was deducted from all samples.

Phosphomannose isomerase (PMI) activity was determined according to Gracy and Noltmann (1968b). Plant leaf material was crushed in liquid nitrogen and 1 ml of extraction buffer (100 mM triethanolamineHCl buffer, pH 7.6 at 25°C, 0.1 % 2-mercaptoethanol) was added. Samples were centrifuged at 13000 *g* at 4°C for 15 minutes. The reaction mix contained 8.7 mM triethanolamineHCl buffer, pH 7.6, 0.55 mM D-mannose-6-phosphate, 0.045 mM nicotinamide adenine dinucleotide phosphate (NADP), 2 units phophoglucoseisomerase, and

0.1 unit glucose-6-phosphate dehydrogenase. Absorption was monitored in a 96 well microplate at 340 nm until stable, and 100 µl of the enzyme extract was added to monitor NADP reduction for 10 min (ε=6.2 mM^{-1} cm^{-1}).

Statistical analyses

Data were analyzed by a two-way ANOVA with the factors treatment, genotype, sampling date, treatment x genotype interaction and treatment x genotype x day interaction (IBM SPSS Statistics 20). Pair-wise genotypic differences in gene expression were determined by post-hoc comparison using the LSD-Test and Tukey adjustment was used for multiple comparison of means if appropriate.

1. 4 Results

Stress responses of contrasting genotypes

Leaf samples were taken on three different sampling days representing different stages of stress. First samples were taken two days after the start of treatments, when the plants showed no symptoms of Zn deficiency. After 16 days (second sampling day), symptoms of Zn deficiency first appeared in IR74, and on the third sampling day after 24 days of treatment, IR74 showed severe stress symptoms as indicated by high leaf bronzing scores (Fig. 2). Shoot Zn concentrations decreased significantly under Zn deficiency and were even lower in the tolerant genotype RIL46 (Fig. 2).
MDA concentrations in leaves increased gradually in IR74 under Zn deficiency, but not in RIL46 (Fig. 2). Together, these data confirmed enhanced Zn deficiency tolerance in RIL46 despite even lower Zn concentration in leaves.

RIL46 showed significantly higher average AsA concentrations in leaves than IR74 (Fig. 3). Total and reduced AsA concentrations declined in IR74 under Zn deficiency starting from day 16 after the beginning of treatments, while the opposite trend was seen in RIL46. IR74 had a 24 % lower total AsA concentration than RIL46 under Zn deficiency after 24 days of treatment. Our further analyses aimed at explaining the contrasting trends in AsA levels in the two genotypes by monitoring (i) AsA biosynthesis and (ii) AsA recycling and degradation.

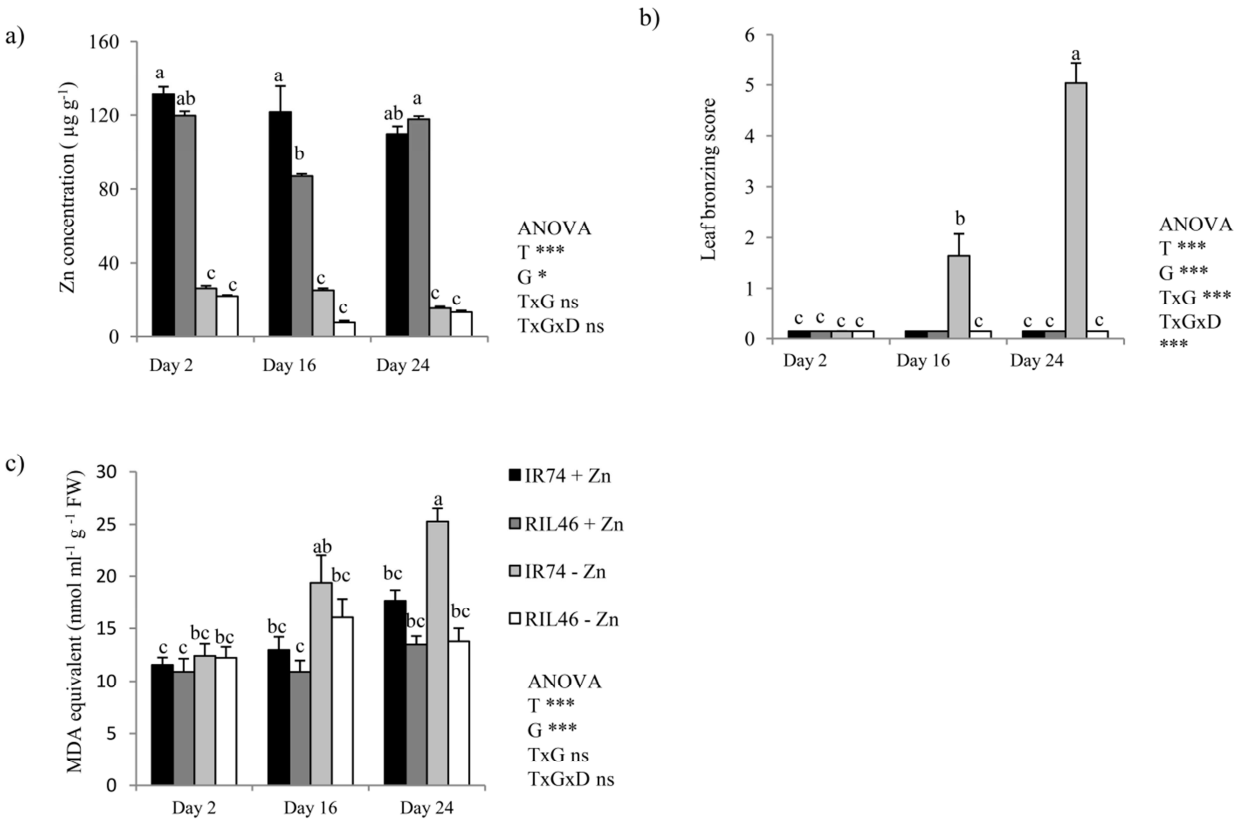

Fig. 2 Influence of zinc deficiency on stress indicators in contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 2, 16, and 24 days of + and −Zn treatments. **a** Shoot Zn concentration of the two genotypes (n = 4). **b** Leaf bronzing score (LBS) (n = 10). **c** Malondialdehyde (MDA) equivalent (n = 4). *T* treatment, *G* genotype, *D* sampling day. ANOVA significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *ns* not significant. *Data bars* not sharing the *same superscript letter* are statistically different at *P* < 0.05 by Tukey's test. *Error bars* indicate standard errors.

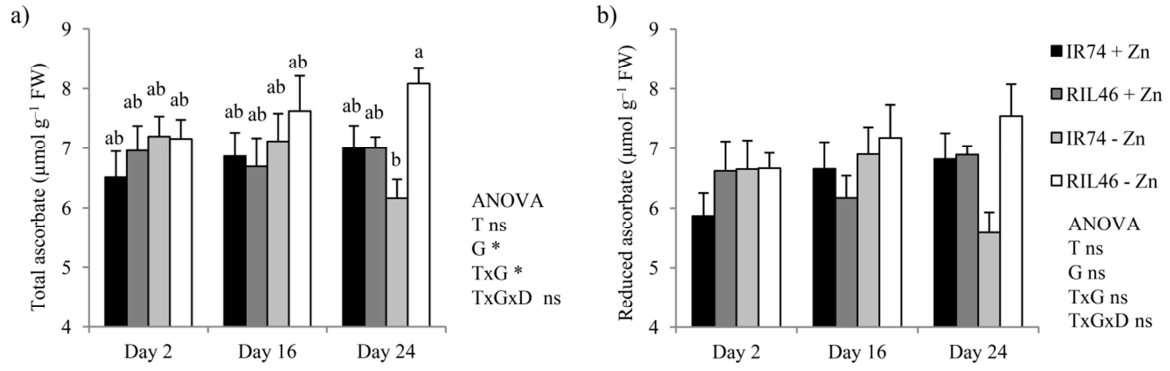

Fig. 3 Influence of zinc deficiency on total and reduced ascorbic acid concentrations in shoots of contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 2, 16, and 24 days of + and – zinc treatments. ANOVA significance levels: * *P*<0.05, ** *P*<0.01, *** *P*<0.001, n.s. not significant. Data bars not sharing the same superscript letter are statistically different at *P*< 0.05 by Tukey test. Error bars indicate standard errors (n=6)

AsA biosynthesis

AsA biosynthesis was analyzed by monitoring the expression of AsA biosynthesis genes (Table 1), selected enzyme activities (Fig. 4), and the concentrations of AsA precursors (Table 2). Pairwise comparison of gene expression levels showed that eight out of twelve genes of the mannose/L-galactose-pathway had higher expression levels in RIL46 as compared to IR74 on at least one sampling day. No consistent trend was seen in gene regulation in either genotype in response to Zn deficiency. Thus, the decline of the AsA pool in the sensitive line cannot be explained by stress-induced down-regulation of AsA biosynthetic genes. The activity of PMI was determined, because it is a Zn-dependent enzyme of the mannose/L-galactose-pathway. The activity was lower under Zn deficiency in both genotypes (Fig. 4), especially on the second sampling day, which was different from PMI gene expression levels (Table 1). In the case of MIOX, the major gene in putative alternative *myo*-inositol pathway, significant differences were observed between the genotypes (Table 1). RIL46 showed significantly higher expression on all the sampling days in the –Zn treatment.

Furthermore, the concentrations of AsA precursors were analyzed (Table 2). Glucose concentration decreased due to Zn deficiency in both genotypes, but was significantly higher in the tolerant RIL46 averaged over all treatment and sampling days. The concentration of phosphorylated intermediates of the mannose/L-galactose–pathway was 2-3 orders of magnitude lower than that of glucose, perhaps indicating their high turnover rates. The concentrations of all measured compounds tended to decrease after 16 days of –Zn treatment, although the treatment effect was significant only for glucose-6-phosphate and mannose-1-phosphate. Significant genotype effects were seen in fructose-6-phosphate and GDP-mannose, where the average concentrations were higher in the intolerant IR74. Additionally, we measured galactose concentration (Supplementary Table S2), but it was analytically not possible to separate D-galactose from L-galactose. The mannose/L-galactose-pathway uses L-galactose as a precursor, while D-galactose serves as a component of the cell wall and of various sugars (French 1954). Thus the increase in total galactose in response to low Zn, especially in the sensitive IR74 (Supplementary Table S2), cannot be ascribed to AsA biosynthesis.

Table 1 Influence of zinc deficiency on gene expression levels of annotated gene isoforms of ascorbate biosynthetic pathways in contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 2, 16, and 24 days of + and −Zn treatments. T, treatment; G, genotype; D, day. Expression of the target genes is normalized by the expression of the internal reference gene Os05g0564200 (RAP-ID, annotated as U2 snRNP). Expression levels are expressed as fold-changes relative to the calibrator IR74 under control conditions on the first sampling day. Color shading indicates significant differences between the genotypes on the same sampling day and treatment: blue indicates lower expression, red indicates higher expression. ANOVA significance levels: light red/blue/*, P<0.05, red/blue/**, P < 0.01, dark red/blue/***, P <0.001, white; not significant (n=4)

		IR74			IR74			RIL46			RIL46			ANOVA			
		+ Zn			-Zn			+Zn			-Zn						
Gene	Locus	Day 2	Day 16	Day 24	Day 2	Day 16	Day 24	Day 2	Day 16	Day 24	Day 2	Day 16	Day 24	T	G	TxG	TxGxD
Phosphomannose isomerase	Os01g0127900	1.0	0.5	0.7	0.6	1.2	0.6	1.1	0.5	0.8	0.8	1.4	1.7	***	***	***	*
GDP-Mannose pyrophosphorylase	Os01g0847200	1.0	0.8	1.4	1.0	0.6	0.9	1.6	1.4	1.6	1.7	1.5	1.9	n.s	***	n.s	
GDP-Mannose pyrophosphorylase	Os08g0237200	1.0	1.1	1.2	0.4	1.2	1.1	0.9	0.4	0.6	2.0	0.6	0.5	n.s	***	n.s	**
GDP-Mannose pyrophosphorylase	Os03g0268400	1.0	0.3	0.4	0.5	1.1	1.3	0.8	0.8	1.1	3.3	1.2	1.5	*	n.s.	n.s	n.s
GDP-mannose 3.5-epimerase	Os10g0417600	1.0	0.6	0.9	0.4	1.0	1.1	1.0	0.7	0.8	0.5	0.9	1.5	n.s	n.s.	n.s	n.s
GDP-mannose 3.5-epimerase	Os11g0591100	1.0	1.2	1.0	0.7	3.7	1.8	1.2	1.1	1.5	1.6	3.0	2.0	n.s	***	n.s	**
GDP-L-galactose phosphorylase	Os12g0190000	1.0	1.2	2.4	1.2	0.9	1.9	1.9	1.3	2.2	1.8	1.4	2.2	n.s	*	n.s	n.s
GDP-L-galactose phosphorylase	Os01g0901300	1.0	1.1	0.9	0.6	1.3	1.3	1.1	0.9	1.3	1.4	1.0	1.7	n.s	*	n.s	n.s
L-galactose-1P-phosphatase	Os03g0587000	1.0	1.0	2.2	1.7	1.2	1.8	0.7	0.8	2.1	2.0	0.6	0.9	**	***	n.s	***
L-galactose dehydrogenase	Os12g0482700	1.0	0.8	0.9	0.6	1.3	1.2	1.3	2.0	1.5	2.6	2.0	2.5	***	***	*	**
L-galactono-1.4-lactone dehydrogenase	Os11g0143500	1.0	1.3	2.0	1.3	0.8	1.3	1.6	2.2	2.0	1.4	3.3	2.0	n.s	***	**	**
L-galactono-1.4-lactone dehydrogenase	Os12g0139600	1.1	0.9	0.8	0.5	1.6	1.8	1.3	2.0	1.1	1.0	2.5	1.3	n.s	n.s		n.s
Myo-inositol oxygenase	Os06g0561000	1.0	1.2	0.8	0.3	0.6	1.0	1.0	0.8	2.1	3.2	4.2	2.9	***	***	***	***

Concentrations of intermediates of the putative alternative *myo*-inositol pathway were also measured (Table 2). No significant differences were seen in glucuronic acid, which showed generally very low levels similar to those of phosphorylated intermediates of the mannose/L-galactose–pathway (Table 2). In contrast, *myo*-inositol exhibited significantly higher average concentrations in the tolerant RIL46. Genotypic differences were particularly pronounced on the third sampling day, when IR74 but not RIL46 showed a decline of *myo*-inositol concentration in the –Zn treatment.

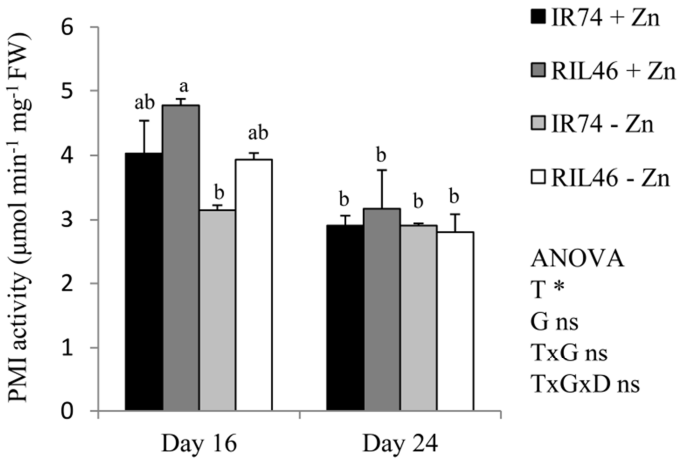

Fig. 4: Influence of zinc deficiency on phosphomannose isomerase (PMI) activity in shoots of contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 16 and 24 days of + and – Zn treatments. ANOVA significance levels: * P<0.05, ** P<0.01, ***, P<0.001, n.s. not significant. Data bars not sharing the same superscript letter are statistically different at P< 0.05 by Tukey test. Error bars indicate standard errors (n=4)

AsA recycling and degradation

We tested DHAR enzyme activity, which showed a slight but significant increase under low Zn stress but no significant genotypic differences (Supplementary Table S2). Other AsA recycling enzymes were not tested as they had already been measured previously in a series of experiments using the same genotypes (Frei et al. 2010b). The glutathione concentration was measured because it is the redox partner in the enzymatic recycling of AsA. The average glutathione concentration was significantly higher in IR74, where it was strongly induced after 16 days of Zn deficiency and decreased again after 24 days of treatment (Fig. 5). RIL46 did not show a similar induction of glutathione, indicating that an elevated glutathione concentration represented an early sensitive response to stress. The amino acid glycine, which constitutes a building block for glutathione biosynthesis, showed a very similar response pattern as glutathione (Fig. 5c). To test whether low Zn stress stimulated AsA degradation, we measured the concentrations of two products of AsA catabolism (Table 2). Unexpectedly, both components tended to decrease under –Zn stress, although the effect was not significant for oxalate. In the case of threonate, the decreases in concentration on days 16 and 24 were more pronounced and highly significant. Genotypic differences did not occur in AsA degradation products.

Table 2 Influence of zinc deficiency on shoot concentrations of ascorbate precursors, degradation products, trehalose and amino acids in contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 2, 16, and 24 days of + and - zinc treatments. T, treatment; G, genotype; D, day. Color shading indicates significant differences between the genotypes on the same sampling day and treatment by pairwise comparison: blue indicates lower concentration, red indicates higher concentration. ANOVA significance levels: light red/blue/*, P<0.05, red/blue/**, P < 0.01, dark red/blue/***, P <0.001, white; not significant (n=5). A complete dataset of all measured metabolites is provided as supplementary data (Table S2)

	IR74			IR74			RIL46			RIL46			ANOVA			
	+ Zn			-Zn			+Zn			-Zn			T	G	TxG	TxGxD
AsA precursors	**Day 2**	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**				
Glucose (nmol g^{-1} FW)	577.5	810.0	813.8	433.0	475.0	912.0	1157.5	1414.0	2042.0	932.5	835.0	1150.0	***	***	**	*
Glucose-6-P (nmol g^{-1} FW)	3.4	3.8	3.6	3.5	2.4	2.5	3.8	3.2	3.3	3.6	1.9	2.1	***	n.s	n.s	n.s
Fructose-6-P (nmol g^{-1} FW)	3.4	3.0	2.9	3.9	1.2	3.5	2.3	0.4	0.9	2.0	0.0	1.4	n.s	***	n.s	n.s
Mannose 1-P (nmol g^{-1} FW)	10.5	11.6	9.6	10.4	10.1	8.0	10.2	11.9	10.0	10.4	7.7	7.7	***	n.s	n.s	n.s
GDP-Mannose (nmol g^{-1} FW)	2.0	1.5	1.3	2.1	1.5	1.1	1.4	1.2	1.3	1.7	0.9	0.7	n.s	***	n.s	n.s
Inositol (nmol g^{-1} FW)	443.5	578.4	803.0	398.0	545.0	448.5	624.3	505.7	635.7	617.6	709.1	647.2	n.s	*	**	n.s
Glucuronic acid (nmol g^{-1}FW)	4.8	7.0	5.7	6.6	4.6	3.9	5.7	8.1	5.8	8.1	5.9	4.2	n.s	n.s	n.s	n.s
AsA degradation products																
Oxalate (nmol g^{-1} FW)	73.8	59.5	148.3	111.1	22.8	52.8	108.7	34.0	66.2	101.1	31.5	40.2	n.s	n.s	n.s	n.s
Threonate (nmol g^{-1} FW)	39.2	52.0	58.6	53.8	14.6	20.7	49.5	52.7	50.4	46.8	12.8	15.5	***	n.s	n.s	n.s
Further metabolites																
Trehalose (nmol g^{-1} FW)	42.0	87.3	69.7	49.8	40.8	24.8	50.5	79.6	76.9	86.5	87.2	68.7	*	***	***	n.s
Selected amino acids																
Serine (nmol g^{-1} FW)	1183.6	1081.1	1840.6	1188.8	8239.2	3165.6	1076.9	1478.7	1297.2	1046.1	4602.6	2802.4	***	***	***	***
Asparagine (nmol g^{-1} FW)	7937.1	1270.5	1519.3	3333.4	8718.3	10883.3	6070.8	1482.7	1811.0	4199.8	5834.2	5542.1	***	***	**	***
Glycine (nmol g^{-1} FW)	407.5	361.7	334.7	359.1	1359.3	808.6	402.4	274.2	291.6	400.1	667.8	685.8	***	***	***	***
Glutamine (nmol g^{-1} FW)	3201.8	869.3	1090.6	1983.1	12749.7	7127.7	2397.2	730.6	903.6	2566.3	7652.7	5043.2	***	***	***	***
Histidine (nmol g^{-1} FW)	51.8	30.5	33.8	46.0	139.6	215.6	48.6	33.8	32.7	43.9	128.9	149.4	***	***	***	***
Arginine (nmol g^{-1} FW)	282.6	91.9	79.2	136.6	1135.0	781.6	232.8	56.8	72.3	96.6	510.4	417.2	***	***	***	*
Proline (nmol g^{-1} FW)	71.1	74.0	85.8	79.0	229.7	174.7	89.4	100.5	102.0	97.0	347.4	805.2	***	***	***	***
Lysine (nmol g^{-1} FW)	94.1	49.8	58.6	73.8	192.3	330.2	76.3	59.9	40.4	53.4	229.1	195.3	***	***	*	***

Further metabolites

Further metabolites were measured that are not directly associated with AsA metabolism, but have been characterized as stress response or tolerance factors in plants. Those compounds may provide additional explanations for genotypic differences in tolerance to Zn deficiency. The antioxidant disaccharide trehalose, which is also involved in sugar signaling/sensing, showed a contrasting response to Zn deficiency in the two genotypes, leading to a significantly higher level in the tolerant RIL46 (Table 2)

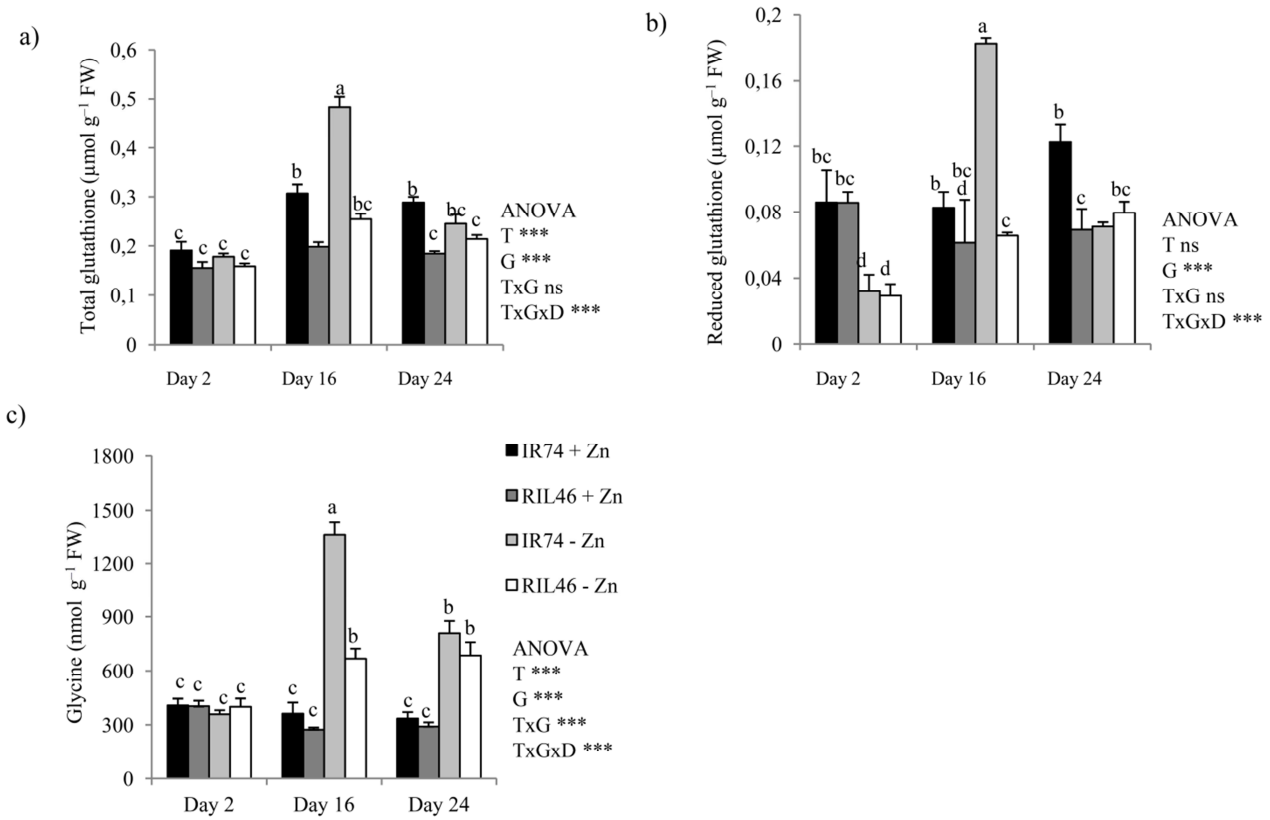

Fig. 5: Influence of zinc deficiency on the concentrations total and reduced glutathione (a,b) and glycine (c) in shoots of contrasting rice genotypes. The susceptible (IR74) and the tolerant (RIL46) rice line were grown hydroponically, and samples were taken after 2, 16, and 24 days of + and -zinc treatments. ANOVA significance levels: * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant. Data bars not sharing the same superscript letter are statistically different at P< 0.05 by Tukey test. Error bars indicate standard errors (n=5)

The concentration of the stress responsive amino acid proline was increased under low Zn stress on the second and third sampling day, but the increase was much more pronounced in the tolerant RIL46 (Table 2). In fact RIL46 had a more than four-fold higher proline concentration after 24 days of –Zn treatment compared to IR74. Thus, the response of proline differed from that of most other amino acids: out of 17 amino acids that showed a significant genotypic difference after 24 days of –Zn treatment, 13 exhibited higher concentration in the intolerant IR74 (Table 2, Supplementary Table S2).

1.5 Discussion

The stress responses of contrasting genotypes observed in this nutrient solution experiment confirm the results of previous experiments in low Zn soil in the Philippines (Wissuwa et al. 2006; Frei et al. 2010b). RIL46 was more tolerant than IR74, although shoot Zn concentrations of both genotypes had to be considered as Zn deficient (Dobermann and Fairhurst 2000). The present study went one step further and detailed the involvement of AsA in preventing oxidative stress under Zn deficiency. By addressing two hypotheses targeted to AsA metabolism, we explored the metabolic basis underlying genotypic differences in AsA metabolism and its involvement in tolerance to Zn deficiency.

Hypothesis 1: Zn deficiency inhibits AsA biosynthesis in the intolerant genotype but not in the tolerant one

The mannose/L-galactose pathway (Fig. 1) is considered as the predominant AsA biosynthetic pathway in plants (Wheeler et al. 1998; Linster and Clarke 2008). Most genes of this pathway were more highly expressed in the tolerant RIL46 on at least one sampling day, in particular the genes at the beginning of the pathway and at the terminal steps, converting L-galactose to AsA. Consistent with these results, a global transcript study by Widodo et al. (2010) reported enhanced expression of three AsA biosynthesis genes (GDP-mannose-3,5-epimerase, L-galactose-1,4-lactone dehydrogenase, L-galactose dehydrogenase) in RIL46, when plants were grown in low Zn soil and samples taken on only one sampling day. Given the complexity of the mannose/L-galactose pathway, it is likely that higher expression levels of single genes in the pathway may not have increased AsA tissue levels. Accordingly, various studies with plants over-expressing only single genes of the mannose/L-galactose pathway did not report any effect on AsA pool size (Bartoli et al. 2005). However, we observed higher expression levels of several genes of the AsA pathway in RIL46, which is indicative for an activation of the whole pathway.

RIL46 had a constitutively higher concentration of glucose (RIL46), which may be due to the ability of RIL46 to maintain enhanced photosynthesis under low Zn stress compared to IR74 (Rose et al 2012). Besides is role as an initial precursor of AsA biosynthesis and many other plant metabolites, glucose is also involved in a complex sugar signaling network, modulating

growth, development and photosynthesis (Rolland et al. 2006) and it may provide a higher energy level for RIL46. Thus, a higher glucose concentration of RIL46 under Zn deficiency may represent an important tolerance factor.

On the other hand, phosphorylated intermediates of AsA biosynthesis showed no significant effects or even higher concentration in IR74, and their availability was generally lower in the –Zn treatments. However, it was suggested that the amount of phosphorylated mannose intermediates is not a limiting factor for AsA biosynthesis (Hancock and Viola 2005). Moreover, the first part of the mannose/L-galactose–pathway is also responsible for producing cell wall polysaccharides, while the second part, subsequent to GDP-L-galactose, is committed only to AsA biosynthesis (Ishikawa et al. 2006). Thus, the early intermediates cannot be interpreted as AsA precursors only.

Compared to the genes of the mannose/L-galactose pathway, an even more pronounced up-regulation in RIL46 was seen for the expression levels of MIOX (Table 1). Additionally, RIL46 exhibited a higher concentration of *myo*-inositol (Table 2), the substrate for this enzyme. However, the contribution of the *myo*-inositol pathway to AsA biosynthesis in plants has been discussed controversially. One study reported a 2-3 fold increase of AsA in MIOX over-expressing *A. thaliana* plants (Lorence et al. 2004), while another study could not confirm these results using the same transgenic lines (Endres and Tenhaken 2009). In a recent study, over-expression of MIOX improved proline concentrations and drought tolerance in rice, although it did not significantly influence AsA pool size (Duan et al. 2012). Thus, the *myo*-inositol pathway is apparently not a major contributor to the AsA pool under normal conditions. Nevertheless, it may provide a backup in the tolerant RIL46 for the mannose/L-galactose pathway, which contains the Zn-dependent enzyme PMI (Gracy and Noltmann 1968a; Maruta et al. 2008). While gene expression of PMI was slightly increased in the –Zn treatment in both genotypes on the second sampling day (Table 1), the enzyme activity was decreased (Fig. 4). This discrepancy may occur because the protein requires Zn to develop its structure and catalytic activity. Whether MIOX is able to contribute to AsA biosynthesis in rice under Zn-deficiency is a hypothesis which remains to be tested using MIOX gene mutants.

In summary, the analysis of the AsA biosynthetic pathways suggests that factors preventing degradation of the AsA level in RIL46 could be higher substrate availability, enhanced expression of some of the biosynthetic genes, and AsA synthesis via the Zn-independent *myo*-

inositol pathway. Limited substrate availability, reduced enzyme activity (especially PMI) and the lack of induction of the *myo*-inositol pathway may be the cause for the decline of the AsA pool in the sensitive line IR74.

Hypothesis 2: Insufficient AsA recycling leads to degradation of AsA in the intolerant genotype, but not in the tolerant one

This hypothesis implies that Zn deficiency causes excessive production of ROS exhausting the recycling capacity of the AsA-glutathione cycle, and consequently leads to catabolism of DHA and accumulation of products such as oxalate and threonate. The recycling enzyme DHAR influenced the AsA poolsize in transgenic gain-of-function mutants of tobacco and maize (Chen et al. 2003; Naqvi et al. 2009). In our study the activity of this enzyme was slightly increased by Zn deficiency, but no genotypic differences were observed (Supplementary Table S2). This is in agreement with a previous study, in which all enzymes of the AsA-glutathione cycle were measured in the same genotypes as used in this experiment, and none of them showed enhanced activity in RIL46 (Frei et al. 2010b). Glutathione forms the redox partner of AsA in the AsA-glutathione cycle (Noctor and Foyer 1998). Its concentration and its precursor glycine (Noctor and Foyer 1998) were strongly induced by Zn deficiency on the second sampling day in IR74, but were almost unaffected in RIL46 (Fig. 5). This pattern is congruent with the stress response concept proposed by Tausz et al. (2004), in which a first initial response of the glutathione system is followed by an acclimation step, leading to a new steady-state. This acclimation step may be represented in the present study by the second sampling day in IR74 (Fig. 5), because glutathione concentration increased in parallel with the stress level. A continuously high level of stress subsequently may have caused degradation of the glutathione-dependent redox system, which was represented by decreased glutathione concentrations on the third sampling day. Therefore, we suggest that high glutathione concentrations reflect a typical early stress indicator but do not significantly contribute to tolerance.

If DHA is not recycled to AsA by enzymatic reduction, it is catabolized to compounds such as oxalate and threonate (Parsons et al. 2011). Some evidence exists that these catabolic pathways also exist in rice (Guo et al. 2005). As opposed to the above-mentioned hypothesis, a decrease instead of an increase in these products of AsA catabolism was found in the –Zn treatment. In other words, plants (especially the sensitive genotype) stimulated AsA recycling, but there was no indication of DHA catabolism due to exhaustion of the recycling capacity.

As we do not find evidence for our second hypothesis we conclude that AsA catabolism is unlikely to contribute to the decline of the AsA pool under low Zn stress.

Other tolerance factors

AsA is the most abundant antioxidant in plants (Noctor and Foyer 1998). The genotypic differences in AsA metabolism (especially biosynthesis) elaborated above can therefore partly explain the differences in oxidative stress tolerance under Zn deficiency. However, our metabolic profiling revealed that other compounds involved in plant stress tolerance also showed genotypic differences, which may have contributed to tolerance in RIL46.

RIL46 maintained a high trehalose level under Zn deficiency, while IR74 showed a constantly lower level under Zn deficiency (Table 2). The antioxidant disaccharide trehalose protects membranes and macromolecules under drought stress as a compatible solute (Rontein et al. 2002), and conferred tolerance to multiple stresses in rice (Garg et al. 2002). Plants with higher trehalose content were also able to maintain a higher photosynthesis level, which is consistent with the proposed role of trehalose as a sugar-sensing and carbohydrate metabolism modulating metabolite (Goddijn and van Dun 1999). There is also evidence that trehalose can scavenge ROS directly under heat stress (Luo et al. 2008). However, it is still under debate if trehalose concentration in higher plants is sufficient to be considered as an active metabolite in stress protection (Wingler and Wingler 2002). Alternatively, the role of trehalose (or rather trehalose-6-P) in sugar signaling may be involved stress reactions and contribute to tolerance (Avonce et al. 2004). Thus, the role of trehalose in stress tolerance should be further explored.

Proline has been well characterized as a stress tolerance factor under salt and drought stress (Ashraf and Foolad 2007). The 5-fold higher concentration in RIL46 under low Zn-stress (Table 2) indicates that it may also be involved in tolerance to Zn deficiency. Apart from its function as an osmo-protectant, previous studies demonstrated a ROS scavenging function of proline (Matysik et al. 2002), and a stabilizing effect on key-enzymes of the AsA-glutathione cycle (Szekely et al. 2008). A number of studies suggested important function of proline in the antioxidant system rather than a factor in osmotic adjustment (Tripathi and Gaur 2004; Molinari et al. 2007). For example, proline reduced lipid peroxidation in transgenic sugarcane plants over-expressing the Δ1-pyrroline-5-carboxylate synthetase gene, which catalyzes the first part of the proline biosynthesis (Molinari et al. 2007). Moreover, over-expression of MIOX in rice induced a higher proline concentration and conferred enhanced tolerance to

drought stress (Duan et al. 2012). Thus, an additional explanation for the tolerance of RIL46 could be a higher MIOX expression leading to an increased proline concentration.

With the exception of proline, alanine and methionine, all amino acids showed either higher accumulation in IR74 under Zn deficiency or no significant difference between genotypes (Table 2). For example, asparagine concentrations were substantially higher IR74 under Zn deficiency, which may indicate inhibited protein synthesis. It was shown earlier that Zn plays a role in regulating protein synthesis (Hossain et al. 1997) and Zn deficient plants accumulated free amino acids, especially asparagine due to impaired protein biosynthesis (Possingham 1956; Kitagishi and Obata 1986; Cakmak et al. 1989). Thus, besides enhanced AsA metabolism, the ability to maintain protein synthesis could be an additional tolerance factor of RIL46 under Zn deficiency.

In conclusion, our data suggest that the ability to maintain a high AsA pool is linked to tolerance to zinc efficiency in rice. Further experiments with AsA mutants will be necessary to determined whether differences in AsA metabolism are a cause or an effect of zinc efficiency. Moreover, the role of other putative tolerance factors such as sugar signaling (trehalose) or proline and their interaction with the AsA metabolism need to be further explored.

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Chapter 2: Zinc deficiency differentially affects redox homeostasis of rice genotypes contrasting in ascorbate level

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2.1 Summary

Zinc (Zn) deficiency is an important mineral disorder affecting rice production, and is associated with the formation of oxidative stress in plant tissue. In this study we investigated processes of oxidative stress formation as affected by ascorbate (AsA) in two pairs of contrasting rice genotypes: (i) Two *indica* lines differing in field tolerance to Zn deficiency and AsA metabolism, *i.e.* RIL46 (tolerant) and IR74 (sensitive); (ii) the *japonica* wild-type Nipponbare (tolerant) and the AsA deficient TOS17 mutant line ND6172 (sensitive) having a 20-30 % lower AsA level due to the knock-out of an AsA biosynthetic gene (*OsGME1*). Plants were grown hydroponically under + and – Zn conditions for 21 days and samples were investigated after 7, 14, and 21 days of treatment. Tissue Zn concentrations below 20 mg kg^{-1} in the –Zn treatment induced the formation of visible symptoms of Zn deficiency from day 14 in all genotypes, but especially in the sensitive IR74. Significant increases in lipid peroxidation were observed in the leaves of the sensitive genotypes IR74 and ND6172, and in the roots of IR74, but not in the tolerant genotypes. At day 21, the tolerant genotypes RIL46 and Nipponbare had significantly higher AsA levels in both shoots and roots compared to the sensitive lines. Consistently, higher levels of hydrogen peroxide formation in leaves and roots of the sensitive genotypes were detected using staining methods. Differences in foliar hydrogen peroxide formation between IR74 and RIL46 became apparent at day 7 and between ND6172 and Nipponbare at day 14. Similarly, genotypic differences in hydrogen peroxide formation in the roots were seen at day 21. In conclusion, our data demonstrate that Zn deficiency leads to a redox imbalance in roots and shoots prior to the occurrence of visible symptoms, and that the antioxidant AsA plays an important role in maintaining the redox homeostasis under Zn deficiency.

Key words: Antioxidants, Zinc deficiency, Reactive oxygen species, *Oryza sativa*, Oxidative stress, TOS17

2.2 Introduction

Zinc (Zn) deficiency is a widespread abiotic stress factor affecting nearly 50 % of soils used for cereal cultivation (Cakmak, 2008). Rice is strongly affected by Zn deficiency as it is often grown in soils characterized by high bicarbonate concentrations, high pH, and low redox potential, factors that are known to influence the amount of Zn present in the soil solution and thus availability to the plants (Alloway, 2009; Sharma et al., 2013). Zn deficiency causes severe yield losses due to reduced growth and development of plants (Alloway, 2004; Quijano-Guerta et al., 2002) and leads to the production of low Zn crops with limited nutritive value in human diets (Hotz and Brown, 2004). On a physiological level, Zn deficiency leads to an imbalance of the production of reactive oxygen species (ROS) and their removal via antioxidants in plant tissue (Cakmak and Marschner, 1988; Cakmak and Marschner, 1993). The resultant 'oxidative stress' leads to necrotic lesions which have been termed as 'leaf bronzing'.

The formation of oxidative stress under Zn deficiency is very complex because Zn has numerous functions in plant cells including processes of gene expression and cell membrane formation, as well as protein metabolism (Marschner and Marschner, 2012). It plays an important role as the catalytic centre of numerous enzymes, including enzymes that are involved in redox processes such as Cu/Zn superoxide dismutase (SOD) (Broadley et al., 2007; Cakmak, 2000) or as a cofactor for metall-containing enzymes, such as violaxanthin de-epoxidase, an important enzyme for non-photochemical quenching (NPQ) during photosynthesis (Müller-Moulé et al., 2002). Moreover the elemental and metabolite profile of plants is greatly affected by the Zn status (Foroughi et al., 2014).

Tolerance to Zn deficiency in rice may be achieved via two mechanisms:

(i) Enhanced uptake of Zn via rhizosphere-based processes. A number of studies (Arnold et al., 2010; Rose et al., 2011; Widodo et al., 2010) suggested that the exudation of low-molecular-weight organic acids or phytosiderophores constitute an important root-based tolerance trait, however, it is still not clear, whether these compounds really cause a better acquisition of Zn in rice (Rose et al., 2013).

(ii) ‘Shoot tolerance’, which implies the lack of oxidative stress symptoms despite low shoot Zn concentration. In previous studies on rice using lines from a recombinant inbred population we found that shoot tolerance to Zn deficiency was not correlated with the activity of enzymatic antioxidants but with the foliar concentration of ascorbate (AsA) (Frei et al., 2010), and we further demonstrated that the redox imbalance leading to oxidative stress under Zn deficiency was at least partly due to inhibited AsA biosynthesis (Höller et al., 2014). AsA is an important antioxidant which can detoxify ROS directly or via an enzymatic network of the Asada-Halliwell cycle (Smirnoff, 2000). Its biosynthesis involves enzymes that require Zn such as phosphomannose isomerase (Gracy and Noltmann, 1968). Whether AsA in roots contributes to tolerance by avoiding oxidative stress has not been investigated so far. However, up-regulation of the enzyme ascorbate peroxidase (APX) under low Zn stress in rice roots (Rose et al., 2012) suggests an involvement of AsA in stress response.

This study specifically investigated the role of AsA in the formation of a redox imbalance in rice roots and shoots under Zn deficiency prior to the generation of visible symptoms. We analyzed four different rice lines representing two contrasting pairs in terms of tolerance to Zn deficiency and AsA levels. One pair consisted of an intolerant high yielding variety IR74, and a tolerant recombinant inbred line RIL46. This pair was shown to contrast in tolerance to Zn deficiency both in the field and nutrient solution experiments, (Frei et al., 2010; Höller et al., 2014; Wissuwa et al., 2006), and also differed in AsA biosynthesis and metabolism (Höller et al., 2014). Another pair consisted of the standard rice genotype Nipponbare (NB), and a TOS17 knock-out mutant in NB genetic background lacking expression of the AsA biosynthesis gene *Os10g041760* (*OsGME1*) encoding a GDP-D-mannose-3`,5`-epimerase (GME). This mutant was shown to contain about 30 % less AsA compared to its wild-type (Frei et al., 2012), and had not been previously characterized regarding its sensitivity to Zn deficiency. Using these two pairs of rice genotypes we tested the hypotheses that: (i) Zn deficiency leads to ROS accumulation and lipid peroxidation in roots and shoots, which precedes the emergence of visible stress symptoms. (ii) AsA is an important factor in maintaining redox homeostasis under Zn deficiency.

2.3 Materials and methods

Plant material

The experiment was conducted with four different rice (*Oryza sativa* L.) lines differing in Zn efficiency and AsA levels. The Zn-efficient recombinant inbred line RIL46 and its intolerant parent IR74 had previously shown contrasting tolerance when grown on low Zn soil in the field (Frei et al., 2010). Seeds were obtained from the Japan International Research Institute for Agricultural Sciences (JIRCAS, Tsukuba, Japan). Additionally, a TOS17 insertion mutant (ND6172) for the gene *Os10g041760* (*OsGME1*) was used, which had previously been characterized regarding its response to tropospheric ozone (Frei et al., 2012). The mutant contains a single insertion in the exon of a gene encoding for a GME leading to complete absence of the respective mRNA (Frei et al., 2012). GME catalyzes two reactions in the biosynthesis of AsA. One is the conversion of GDP-D-mannose to GDP-L-galactose in the predominant pathway (Wheeler et al., 1998), and the other is the conversion of GDP-D-mannose to L-gulose in an alternative pathway (Wolucka and Van Montagu, 2003). Seeds were originally obtained from the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (Hirochika, 2010). The wild-type background of this mutant, the *japonica* variety NB, was used as control.

Experiment

A hydroponic experiment was conducted in a glasshouse with controlled minimum night/day temperatures of 22°C/ 28°C and an average relative air humidity of 50 %. Artificial lighting was supplemented from 7 am to 8 pm to ensure a minimum photosynthetic photon flux density (PPFD) of 400 µmol m^{-2} s^{-1}. In total 20 replicates per genotype per treatment were grown. Seeds were germinated on styrofoam sheets at 30°C for 4 days in the dark and transferred to the greenhouse floating on 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$ for another 7 days. Thereafter, seedlings were transferred to 60 L hydroponic tanks filled with half strength nutrient solution containing no Zn. After 7 days, plants were grown on full-strength nutrient solution containing 1 mM $NaHCO_3$ (Rose et al., 2011) and either no Zn (-Zn treatment) or 1 µM Zn (+Zn treatment). Nutrient solutions consisted of 1.42 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.51 mM K_2SO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 9 µM $MnCl_2$, 0.07 µM

$(NH_4)_6Mo_7O_{24}$, 18.5 µM H_3BO_3, 0.16 µM $CuSO_4$ and 35.6 µM $FeCl_3$. The pH of the nutrient solution was adjusted to pH 5.5 twice a week and the nutrient solution was completely exchanged after 10 days. ROS staining of leaves was conducted 7, 14 and 21 days after the start of the treatment, and staining of roots was carried out after 21 days. Whole shoots and roots for biochemical analyses were harvested 14 and 21 days after start of treatment. A leaf bronzing score (LBS) ranging from 0 (healthy leaf) to 10 (dead leaf) was assigned to the three youngest fully expanded leaves of each plant (Wissuwa et al., 2006) and tiller number, shoot height and shoot weight were recorded.

Biochemical analyses

Zn concentration was measured in dried leaves and roots by atomic absorption spectrometry. Roots were washed with deionized water before the harvest to remove residues of nutrient solutions. In case of roots, it was not possible to measure Zn concentration on day 14 because of the limited amount of root material.

Reduced and oxidized AsA was measured immediately after plant harvesting according to Ueda et al. (2013). Shoots were flash frozen in liquid nitrogen and ground to a fine powder. Approximately 80 mg of shoot material were dissolved in 1 mL 6 % metaphosphoric acid (MPA) and 1 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 15000 g at 4°C for 20 minutes. Root material was extracted with 1 mL 6 % trichloroacetic acid (TCA) (Gillespie and Ainsworth, 2007) and centrifuged (15000 g, 4°C, 20 minutes). Supernatants were used for the analyses. The reaction mix contained 10 µL of shoot extract or 20 µL of root extract , 80 mM potassium phosphate buffer (pH 7.0) and 0.1 U ascorbate oxidase (AO). For every sample an additional blank well was added, where the amount of AO was substituted by the same amount of potassium phosphate buffer (pH 7.0). For the measurement of oxidized AsA, the reaction mix contained 10 µL of shoot extract or 20 µL of root extract, 80 mM potassium phosphate buffer (pH 7.8) and 4 mM dithiothreitol (DTT). In blank wells, DTT was substituted by the same amount of potassium phosphate buffer (pH 7.8). For both measurements, absorbance was monitored in a microplate reader (Powerwave XSII, BioTek) at 265 nm in UV-transparent 96-well microplates until it was constant. Total AsA was calculated as the sum of reduced and oxidized AsA.

Malondialdehyde (MDA) was measured according to Hodges et al. (1999). Approximately 80 mg of shoot material and 100 mg of root material was dissolved in 1 mL of 0.1 % TCA and centrifuged at 14000 g at 4°C for 15 minutes. In the case of shoot material, the extraction step

was repeated and the supernatants were combined. 250 µL of reaction solution I (0.01 % (v/v) 2,6-di-tert-butyl-4-methylphenol (BHT) in 20 % TCA (v/v)) was added to the same amount of plant extract, while 250 µL of reaction solution II (0.01 % (v/v) BHT and 0.65 % 2-thiobarbituric acid (TBA) in 20 % TCA (v/v)) was added to another 250 µL of plant extract. Samples were incubated at 95°C for 30 minutes and the reaction was stopped on ice. Samples were centrifuged (8000 g, 4°C, 10 minutes) and absorbance was measured at 440, 532 and 600 nm with a microplate reader.

ROS detection in leaves

For in-situ detection of H_2O_2 in leaves, staining of the first and second youngest fully expanded leaf was carried out with 3,3'-diaminobenzidine (DAB) 7, 14 and 21 days after treatment according to Thordal-Christensen et al. (1997) with some modifications. At least two leaves of four different plants per genotype, per treatment and per sampling day were analyzed. Staining solution was prepared by dissolving 0.5 mg per mL of DAB in water, adjusting the pH to 3.8, and filtering. Leaves were detached, washed with 0.05 % Triton X-100 and rinsed with distilled water. They were then placed in DAB-solution for 12 hours in the dark and afterwards rinsed again with distilled water. Samples were incubated in Glycerol/Lactic acid/Ethanol = 1/1/4 at 85°C for 30-90 minutes to remove chlorophyll and stored in fresh decolorizing solution. H_2O_2 was visualized as brown precipitation, documented by a Leica DM LB light microscope (Leica Microsystems, Wetzlar, Germany) and photographed by a Leica DCF425 camera (Leica Microsystems, Wetzlar, Germany).

Fluorescent staining of ROS in roots

H_2O_2 generation in primary and secondary lateral roots was visualized by the fluorescent probe dihydrodichlorofluorescein diacetate (H_2DCF-DA) 21 days after the start of treatments according to Parlanti et al. (2011) with some modifications. At least four roots per plant and per genotype were analyzed. Roots were incubated for 15 minutes in 10 mM Tris-HCl buffer (pH 7.4) containing 10 µM H_2DCF-DA. To detect living cells, roots were additionally stained with 50 µM propidium iodide (PI) which intercalates with nucleic acids if cell walls are damaged. PI was added immediately before documentation of the fluorescent signal. Afterwards, roots were rinsed with distilled water and placed on a slide in a drop of water. Samples were imaged using a Zeiss confocal microscope LSM 780 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 25x lens (Zeiss Objective C-Apochromat 25×/0.8 W Corr

M27). Fluorescence was excited at 488 nm and emission was recorded at 501-572 nm. Simultaneous labeling of cell walls with PI was recorded at 582-649 nm after excitation at 543 nm.

Statistical analyses

The data were analyzed by analysis of variance (ANOVA) with the factors treatment, genotype and sampling day, treatment x genotype and treatment x genotype x sampling day using the software IBM SPSS Statistics 21. Pair-wise comparison within the same sampling day was conducted using the Fisher's Least Significant Difference (LSD) test.

2.4 Results

Phenotypical and biochemical characterization of stress response

Stress response of the different genotypes to Zn deficiency was examined by biochemical and morphological analyses of roots and shoots as well as different ROS staining techniques. Shoot Zn concentrations decreased under Zn deficiency in all genotypes with no significant genotypic differences (Table 1). Concentrations were even lower under Zn deficiency in the tolerant lines RIL46 and NB, although these differences were not statistically significant. The pattern was partly different in roots (Table 1), where Zn concentrations decreased under Zn deficiency, but remained higher in RIL46. No difference in root Zn concentration occurred between the AsA deficient mutant ND6172 and NB.

All genotypes developed visible symptoms of oxidative stress under Zn deficiency from day 14 after the start of the –Zn treatment, with IR74 showing significantly higher LBS than RIL46 (Table 1, Fig. S1). In contrast, no significant difference in LBS was seen between ND6172 and NB. Although the tolerant lines RIL46 and the NB had a tendency towards increased shoot dry weight under stress conditions, no significant differences were observed. Root length was constitutively higher in IR74 than in RIL46, but shorter root length due to Zn deficiency was observed in IR74 but not in RIL46. No significant genotypic difference in root length was observed between ND6172 and NB (Table 1). Thus, visible phenotypic differences only occurred between IR74 and RIL46, although biochemical analyzes revealed differences in oxidative stress level also between ND6172 and NB. MDA levels in shoot tissue increased in all genotypes under stress conditions, but statistically significant increases were seen only in the sensitive genotypes IR74 and ND6174 (Fig. 1a, b). The amount of MDA in roots was much lower than in shoots and the differences between control and stressed plants were not as distinct as in shoots (Fig. 1c, d). However, both sensitive genotypes IR74 and ND6172 showed a constitutively higher level of root MDA and a significant increase due to Zn deficiency was noted in IR74 but not in the other genotypes.

Table 1: Phenotypic response to zinc deficiency of two pairs of rice genotypes contrasting in ascorbate concentration: RIL46/IR74 (a) and NB/ND6172 (b).

a)

	+ Zinc				-Zinc				ANOVA				
	RIL46		IR74		RIL46		IR74						
	14 days	21 days	14 days	21 days	14 days	21 days	14 days	21 days	T	G	D	TxG	TxGxD
Shoot Zn (mg kg-1)	47,9	30,6	63,3	44,3	23,1	13,9	27,4	14,8	***	ns	**	ns	ns
Root Zn (mg kg-1)	/	48,6	/	37,3	/	21,9	/	14,4	***	***	/	ns	/
LBS	0	0	0	0	0,5	0,7	1,4	2,8	***	***	**	***	ns
Shoot height (cm)	47,0	52,3	44,5	50,3	43,3	49,5	46,0	53,3	ns	ns	***	ns	ns
Shoot weight (g)	1,2	2,3	1,5	3,3	1,7	2,4	1,5	2,1	ns	ns	**	ns	ns
Root size (cm)	16,9	17,2	23,7	25,3	16,5	17,8	19,9	22,2	ns	*	*	ns	ns
Tillers	1,8	3	2,5	2,5	2,3	2,8	2	2,3	ns	ns	ns	ns	ns

b)

	+ Zinc				- Zinc				ANOVA				
	NB		ND6172		NB		ND6172						
	14 days	21 days	14 days	21 days	14 days	21 days	14 days	21 days	T	G	D	TxG	TxGxD
Shoot Zn (mg kg-1)	71	32,5	53,1	35,9	19,9	13,2	21	16,2	***	ns	**	ns	ns
Root Zn (mg kg-1)	/	19,8	/	19,7	/	16,2	/	14,1	***	ns	/	ns	/
LBS	0	0	0	0	0,7	1,2	1	1,1	***	ns	ns	ns	ns
Shoot height (cm)	49,5	64,3	50,8	60,3	51	58,3	49,5	57,8	ns	ns	***	ns	ns
Shoot weight (g)	1,3	3,2	1,5	2,5	1,5	2,4	1,2	1,9	ns	ns	***	ns	ns
Root size (cm)	16,4	18,2	17,5	18,6	17,5	18,2	19,6	20,4	ns	ns	*	ns	ns
Tillers	1,5	2,8	1,8	2,3	1,8	2,3	1,5	1,8	ns	ns	*	ns	ns

T, treatment; G, genotype; D, day; LBS, leaf bronzing score; DW dry weight; nd not determined; Samples were grown with +Zn and –Zn treatment and samples were taken after 14 and 21 days. Root zinc concentration could not be analyzed on day 14 due to insufficient plant material. ANOVA significance levels: *, 0.05, **, 0.01, ***, 0.001, n.s. not significant (n=4).

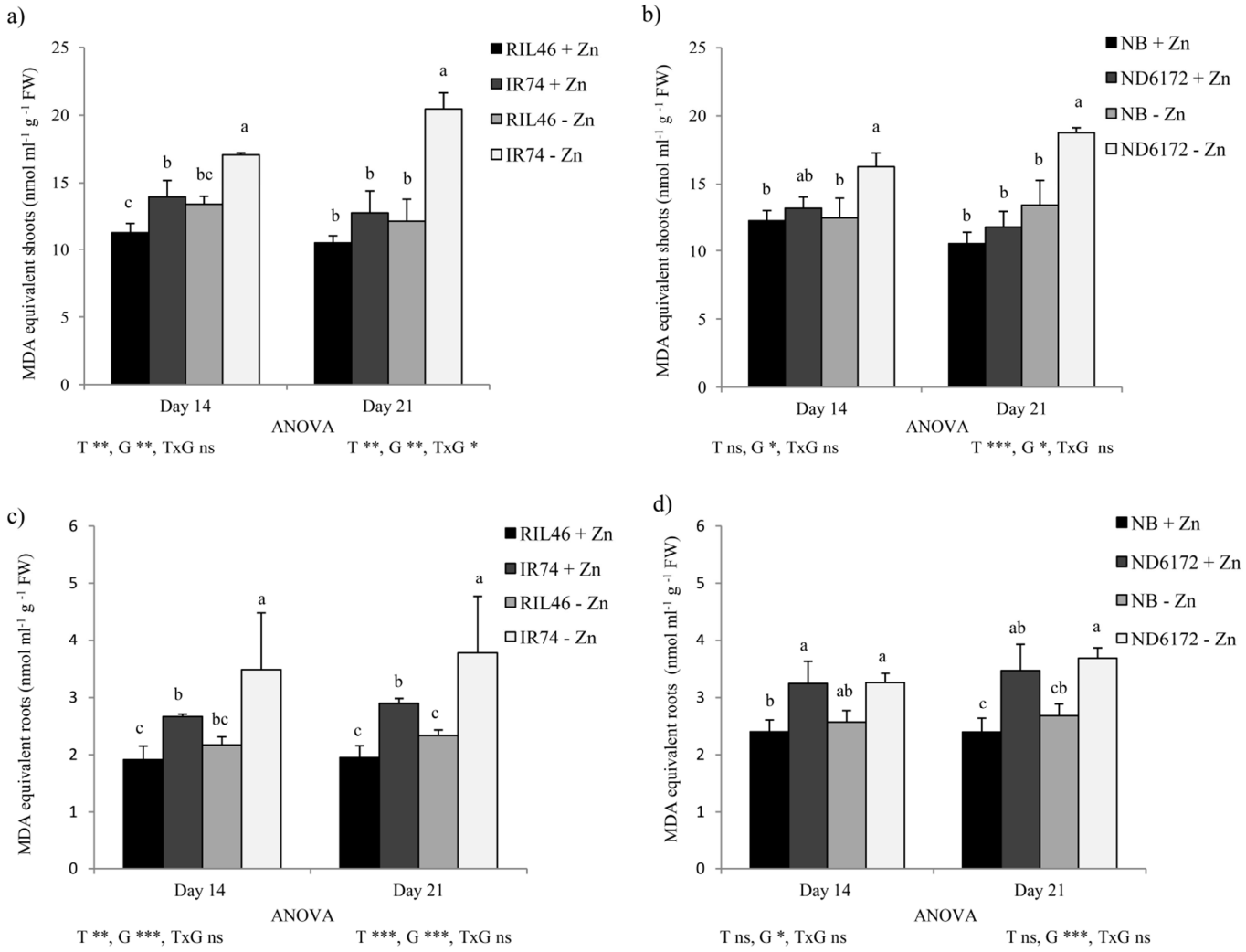

Fig. 1: Malondialdehyde (MDA) concentrations in root and shoots of different rice genotypes under control and zinc deficient conditions on two sampling days. a) Shoot MDA concentration of the contrasting pair RIL46 and IR74 b) Shoot MDA concentration of NB and the mutant line ND6172 c) Root MDA concentration of RIL46 and IR74. d) Root MDA concentration of NB and ND6172. ANOVA significance levels: *, 0.05, **, 0.01, ***, 0.001, n.s. not significant. Data bars not sharing the same superscript letter are statistically different within the same sampling day at $P < 0.05$ by LSD- test. Vertical bars indicate standard errors (n=4).

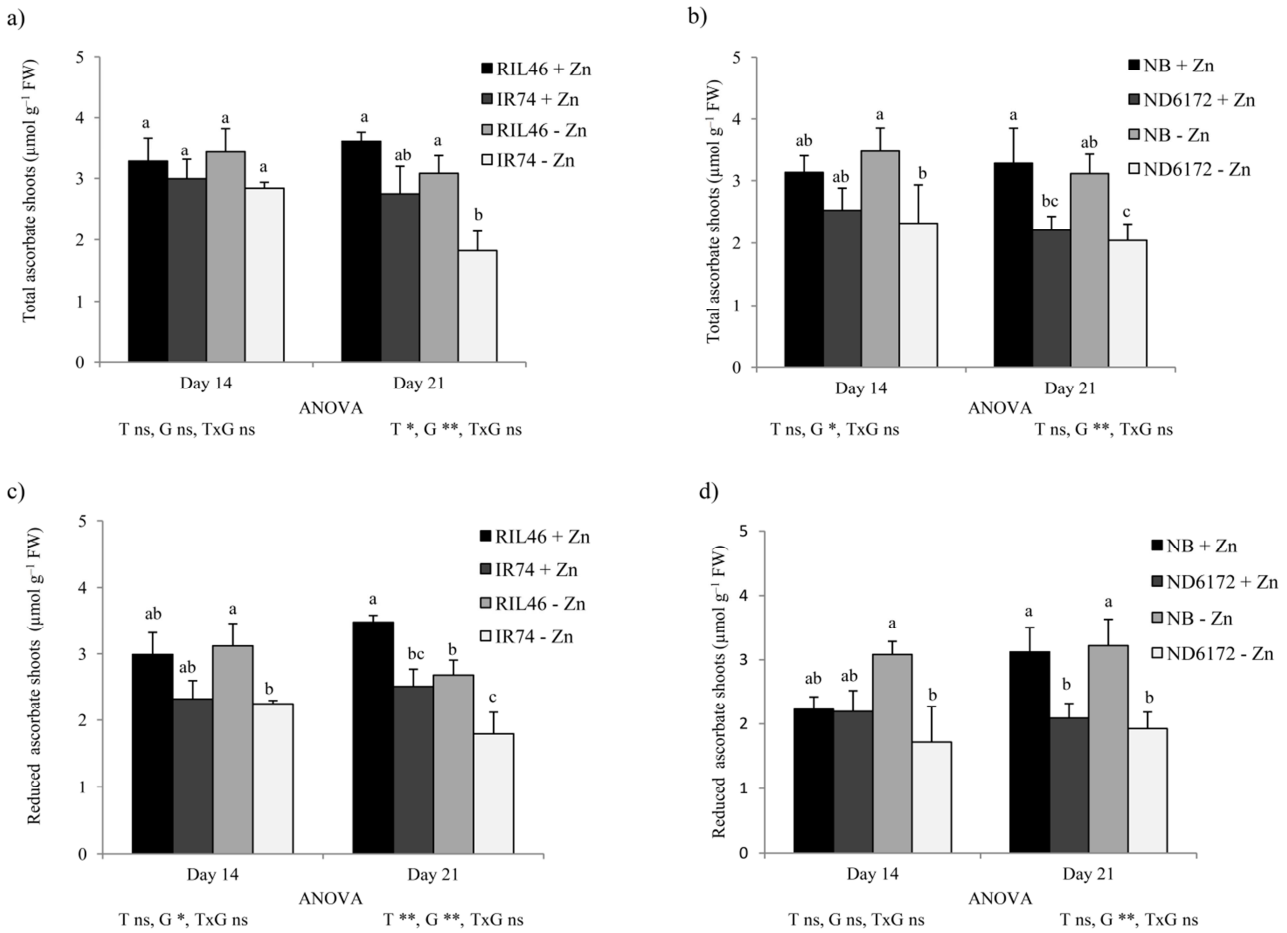

Fig. 2: Total and reduced shoot ascorbate concentrations of different rice genotypes under control and Zn deficient conditions on two sampling days. a) Shoot total ascorbate concentration of the contrasting pair RIL46 and IR74. b) Shoot total ascorbate concentration of NB and the mutant line ND6172. c) Shoot reduced ascorbate concentration of the contrasting pair RIL46 and IR74 d) Shoot reduced ascorbate concentration of NB and the mutant line ND6172. ANOVA significance levels: *, 0.05, **, 0.01, ***, 0.001, n.s. not significant. Data bars not sharing the same superscript letter are statistically different within the same sampling day at $P < 0.05$ by LSD- test. Vertical bars indicate standard errors (n=4).

Total AsA decreased in shoots of IR74 (especially after 21 days of Zn deficiency) compared to RIL46, which showed generally higher AsA levels and only little decrease under Zn deficiency (Fig. 2a, b). IR74 had a 17 % lower AsA concentration on day 14 under Zn deficiency compared to RIL46, and a 41 % lower AsA level on day 21. The mutant line ND6172 had a consistently lower AsA level (about 30 %), even under control conditions, compared to its wild-type NB. A slightly different effect was seen in the roots (Fig. 3). AsA concentrations were about 70 % lower than in shoots in all genotypes. Differences in AsA

concentration between ND6172 and NB were similar to the shoots (20-30 %), while IR74 had about 40-50 % less AsA than RIL46 under Zn deficiency. About 80-90 % of total AsA was present in its reduced state with no differences between roots and shoots and no treatment effect (Figs. 2 c, d and 3 c, d).

Taken together, phenotypic symptoms reflected the oxidative stress level of IR74 better than in ND6172, which did not differ in phenotypic symptoms from its wild-type, but nevertheless had significantly higher level of oxidative stress due to lower AsA concentrations.

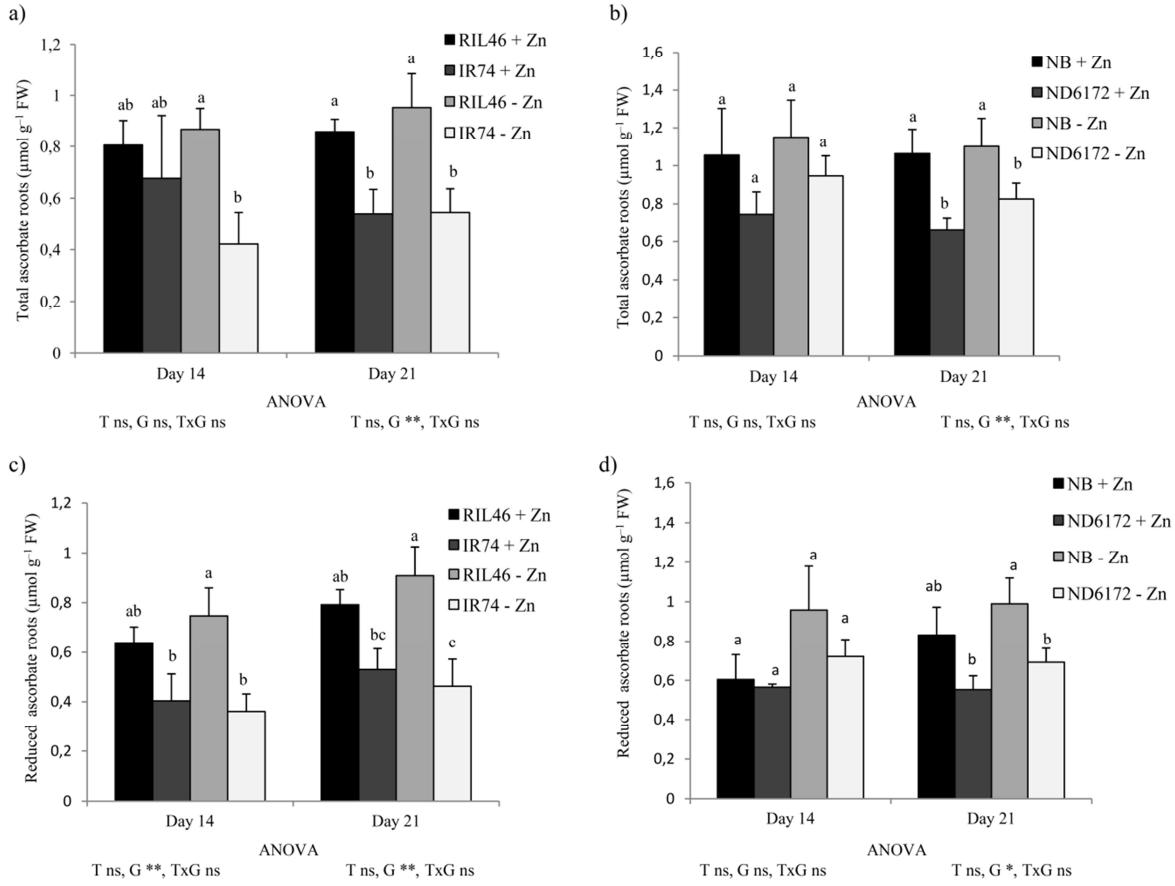

Fig. 3: Total and reduced root ascorbate concentrations of different rice genotypes under control and zinc deficient conditions on two sampling days. a) Root total ascorbate concentration of the contrasting pair RIL46 and IR74. b) Root total ascorbate concentration of NB and the mutant line ND6172. c) Root reduced ascorbate concentration of the contrasting pair RIL46 and IR74d) Root reduced ascorbate concentration of NB and the mutant line ND6172. ANOVA significance levels: *, 0.05, **, 0.01, ***, 0.001, n.s. not significant. *Data bars* not sharing the *same superscript letter* are statistically different within the same sampling day at $P < 0.05$ by LSD- test. Vertical bars indicate standard errors (n=4).

Staining of ROS in leaves

In addition to biochemical characterization of oxidative stress in the plants we used histochemical staining methods to examine the localization and intensity of ROS production *in situ*. Staining of H_2O_2 in the first and second youngest fully expanded leaf of all genotypes was performed after 7, 14 and 21 days of Zn deficiency using a DAB-assay. Although there were no visible symptoms of oxidative stress in IR74 under Zn deficiency after 7 days (not shown), DAB-staining already indicated an increased H_2O_2 level (Fig. 4). IR74 was the only genotype already developing an increased ROS level after 7 days, while the mutant line ND6172 showed increased staining of leaves after 14 days of treatment compared to its wild-type NB. After 21 days of treatment, all genotypes showed clearly increased H_2O_2 production compared to controls, while IR74 showed the most severe H_2O_2 accumulation. Generally, brown precipitations could be observed in mesophyll cells, except for vascular tissue of RIL46, the only genotype showing clearly visible staining of the stele (Fig. 4a, b). Together with the other indicators of oxidative stress such as LBS and MDA, the staining of H_2O_2 with DAB provided evidence of unbalanced redox homeostasis in leaves prior to the generation of visible symptoms. Leaves were only stained with DAB and not with the fluorescent dye H_2DCF-DA because of poor uptake of the dye and hence unreliable results.

Staining of ROS in roots

Roots of all genotypes were stained with the fluorescent dye H_2DCFDA after 21 days of treatment to visualize H_2O_2 generation. The strongest fluorescent signal was seen in roots of ND6172 under Zn deficiency (Fig. 5a). The tolerant lines RIL46 and NB emitted lower fluorescence signals in both control and Zn deficient conditions. Similar to the MDA measurements in roots, the treatment effect on ROS formation in roots was not as pronounced as in the leaves. Interestingly, a stronger staining of the central cylinder was observed under Zn deficiency in all genotypes. While the signal was constitutively stronger in IR74 and ND6172, it shifted from the epidermal cells to the central cylinder only in RIL46 and NB, the more tolerant genotypes. To verify the integrity of the root cells and to show that H_2O_2 production was caused by Zn deficiency and not by ROS leakage due to sample damage, cells were additionally stained with PI. Staining was detected only in the cell walls and not in nucleus (Fig. 5b), indicating that H_2O_2 production was not caused by mechanical damage of the cells.

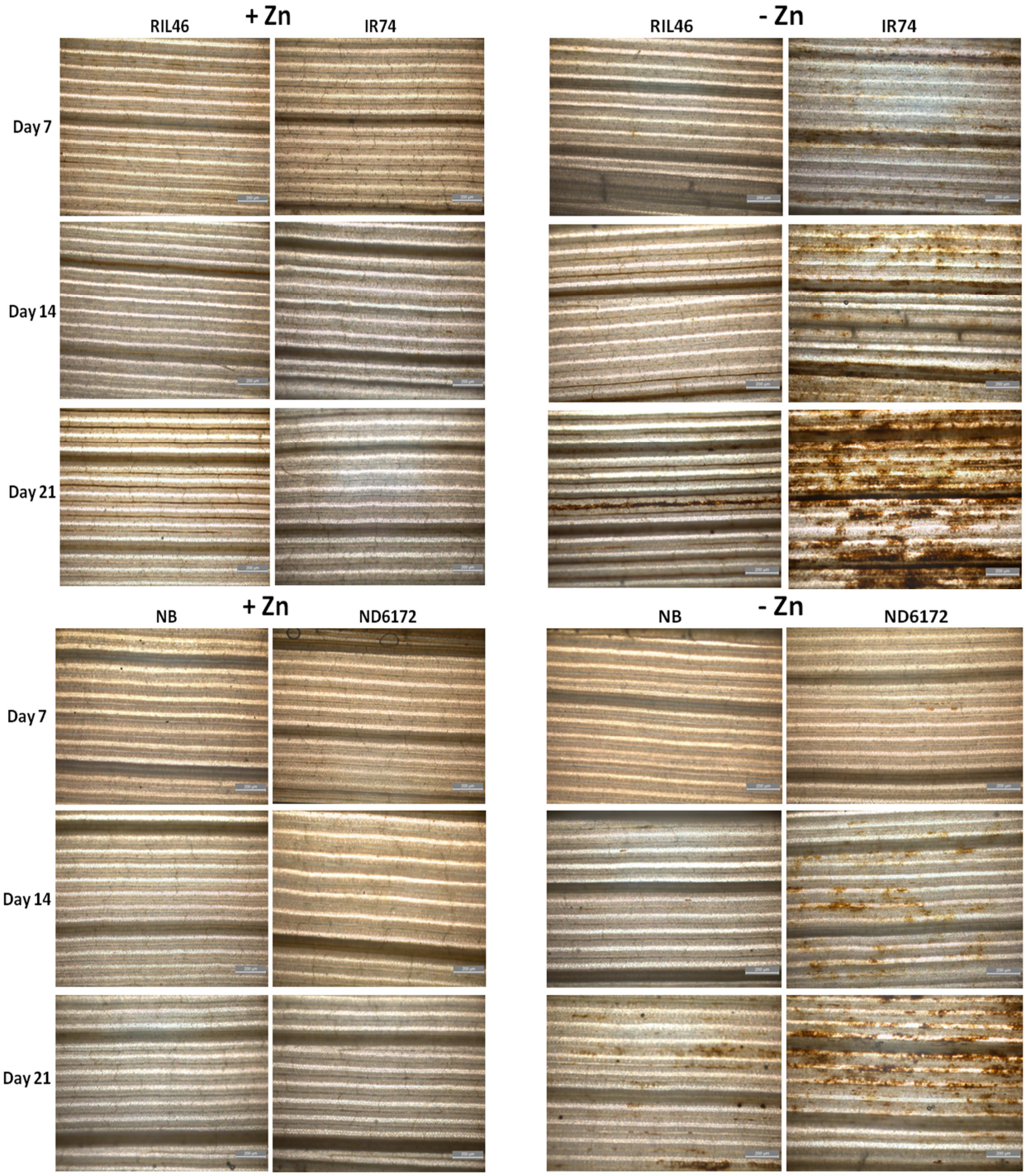

Fig. 4. Microscopic detection of H_2O_2 in rice leaves of genotypes differing in zinc deficiency. Leaves were stained with 3,3`-diaminobenzidine (DAB) 7, 14 and 21 days after +zinc and −zinc treatment. In total, two leaves of four plants per treatment and genotypes were stained and representative images are shown. (a) DAB staining in control leaves ofRIL46 and IR74 at three different sampling days. (b) DAB staining in leaves of RIL46 and IR74 under zinc deficiency at three different sampling days. (c) DAB staining in controlleaves of NB and the mutant line ND6172 at three different sampling days. (d) Leaves of NB and ND6172 under zinc deficiency at three different sampling days.

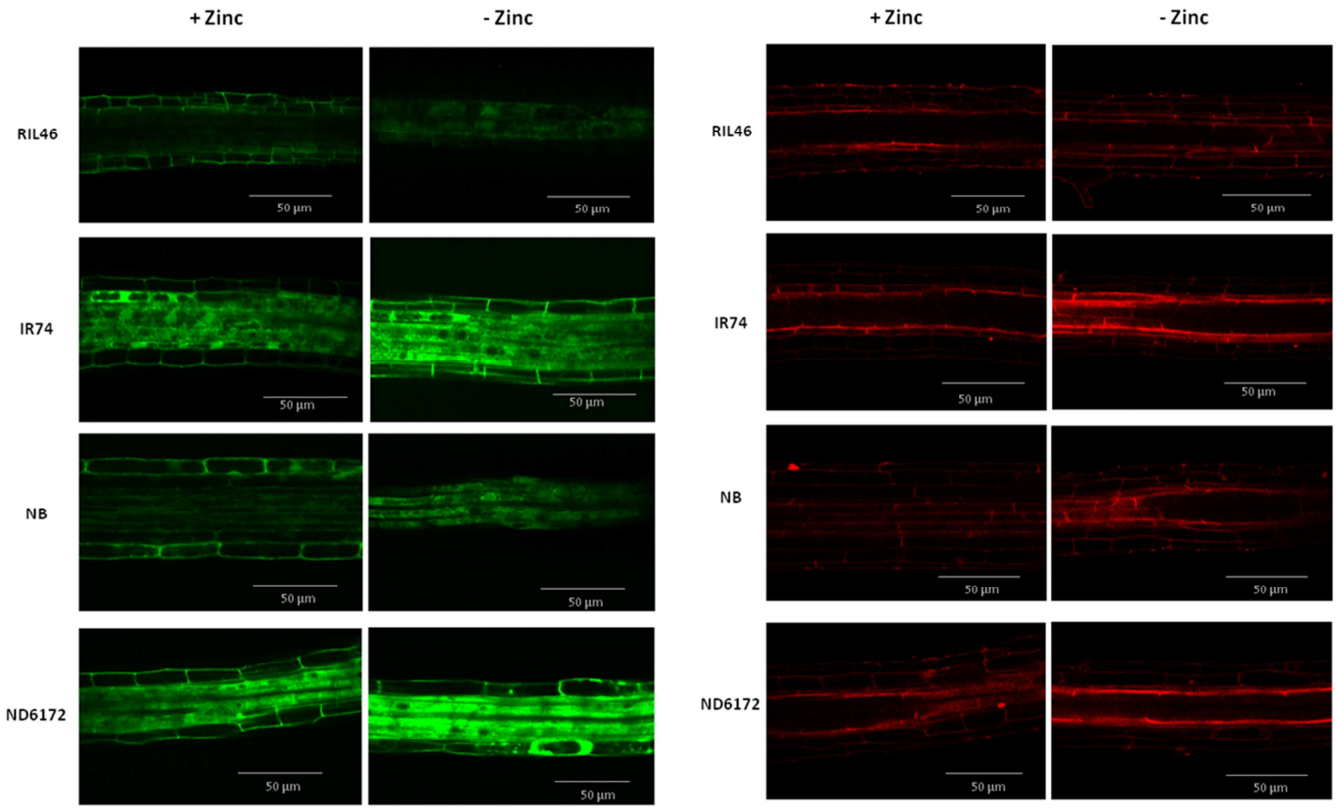

Fig. 5. (a) Staining of reactive oxygen species in roots using the fluorescent dye H_2DCFDA to visualize the impact of zinc deficiency and different ascorbate concentrations.(a) Second order lateral roots of RIL46/IR74 and NB/ND6172 were stained with H_2DCFDA 21 days after +zinc and −zinc treatment. (b) Roots were additionally stained withpropidium iodide to confirm the integrity of the cells.

2.5 Discussion

Leaf bronzing is a typical symptom of Zn deficiency in rice (Wissuwa et al., 2006). In this study we aimed to unravel processes leading to the formation of oxidative stress and consequently leaf bronzing symptoms by addressing two specific hypotheses.

Hypothesis 1: Zn deficiency leads to excessive ROS formation in roots and shoots resulting in visible damage

Zn deficiency clearly induced oxidative stress in our plants as represented by H_2O_2 production and followed by increased lipid peroxidation (Figs. 1, 4, 5). Several studies addressed the question how lack of Zn induces oxidative stress in plants, and the answer may be complex due to the ubiquitous role of Zn in plant metabolism. Zn is a functional component of thousands of proteins in plants, including the antioxidant enzyme Cu/Zn SOD (Tainer et al., 1983) or the AsA biosynthetic enzyme phosphomannose isomerase (Gracy and Noltmann, 1968). Zn deficiency affected the photosynthetic electron transport and increased the production of the superoxide radical in bean (Marschner and Cakmak, 1989) and maize leaves (Shrotri et al., 1981; Wang and Jin, 2005). Furthermore, Zn can form chelates with many ligands such as cysteine or histidine (Vallee and Auld, 1990) with a greater affinity than iron (Fe). Therefore electron transfer caused by cysteine-bound Fe is inhibited by adequate Zn availability (Bray and Bettger, 1990), while Zn deficiency may foster the formation of Fe-cysteine complexes leading to autocatalytic formation of superoxide via the Fenton reaction. Adequate Zn supply can also inhibit production of the superoxide radical, catalyzed by the enzyme NADPH oxidase (Cakmak and Marschner, 1988; Pinton et al., 1994). Due to the complexity of these simultaneous processes occurring in plants, it is difficult to differentiate between cause and effect in oxidative stress and symptom formation under Zn deficiency. Our results indicate that excessive ROS production precedes the formation of leaf symptoms and biomass reductions. That antioxidant response to oxidative stress occurred prior to visible symptoms of Zn deficiency was also shown for wheat (Sharma et al., 2004), and provides further evidence that the disturbance of redox homeostasis is a major factor of plant stress under Zn deficiency.

Regarding oxidative stress production in the roots, we saw a low ROS signal in all genotypes under control conditions and an increase under Zn deficiency (Fig. 5). Formation of oxidative stress in roots due to Zn deficiency was previously reported in different species such as bean

(Pinton et al., 1994), cotton and tomato (Cakmak and Marschner, 1988). Rhizosphere-related processes such as increased exudation of Zn chelators resulting in higher Zn uptake are considered to be the main root based tolerance factors in rice (Rose et al., 2012). In addition our results indicate that roots exposed to Zn deficiency produce an increased amount of ROS. Contrasting genotypes clearly differed in ROS production under Zn deficiency, suggesting differences in antioxidant capacity may represent an additional tolerance factor. Interestingly, the most intense fluorescent signal in the tolerant genotypes RIL46 and NB was observed in the stele under Zn deficiency (Fig. 5). It is known that lignification of the xylem can occur in conditions of mineral deficiencies, such as silicon deficiency in rice roots (Frei, 2013; Suzuki et al., 2012) or manganese deficiency in wheat (Rengel et al., 1994). As lignification is a redox regulated process (Almagro et al., 2009; Frei, 2013) it may be worthwhile exploring whether Zn deficiency induces lignin biosynthesis due to changes in redox homeostasis, and whether this represents a tolerance factor.

Hypothesis 2: AsA plays an important role in maintaining redox homeostasis under Zn deficiency

In order to cope with excessive production of ROS caused by abiotic stress, plants developed a network of antioxidants. Studies on diverse plant species reported correlations between the concentrations of AsA and oxidative stress levels under different abiotic stresses such as ozone stress in rice (Frei et al., 2012) and *Arabidopsis thaliana* plants (Conklin et al., 1996), or salinity in potato (Hemavathi et al., 2009). In the case of Zn deficiency, a number of studies investigated detoxification of ROS by enzymes such as the Cu/Zn-SOD, which was affected by Zn deficiency in rye and wheat (Cakmak et al., 1997), or APX showing decreased activity in Zn deficient tobacco plants (Yu et al., 1998). In a previous metabolomic study focusing on shoots, we proposed a link between tolerance to Zn deficiency and AsA biosynthesis in RIL46 and IR74 (Höller et al., 2014). This current study confirmed this principle using the mutant line ND6172 lacking about 30 % AsA compared to the wild-type NB (Fig. 2) due to a knock-out of the biosynthesis gene OsGME1, which showed higher sensitivity to low Zn stress despite similar shoot Zn concentration (Figs. 2, 4, 5).

On the root level the main tolerance factor of Zn efficient rice cultivars might be enhanced Zn uptake (Arnold et al., 2010), which may be explained with greater root solute exudation (Rose et al., 2011). Our study confirmed that RIL46 had significantly higher root Zn

concentrations (Table 1). However, such differences in Zn concentration did not occur between the mutant line ND6172 and NB, where a 20-30 % lower AsA level in ND6172 resulted in a higher oxidative stress level in roots (Fig. 3). Our results thus suggest that AsA level was an important tolerance factor not only in shoots but also in roots, where it may complement other tolerance factors such as exudation of Zn chelators. Rose et al. (2012) previously reported that the activity of the enzyme APX, responsible for detoxification of H_2O_2, was increased by Zn deficiency in rice roots. That AsA is involved in oxidative stress response in roots could also be shown for other abiotic stresses such as hypoxia in lupine roots (Garnczarska, 2005) or aluminum toxicity in roots of *Cassia tora* (Wang and Yang, 2005).

AsA concentrations in roots were significantly lower than in shoot (Figs. 2 and 3). Franceschi and Tarlyn (2002) reported that AsA biosynthesis and turnover rate was much higher in photosynthetic tissue, and that AsA might have to be transported from source to sink tissue, which was shown for exogenously applied AsA in three different species *(Arabidopsis thaliana*, *Medicago sativa* and *Impatiens walleriana)*. In that study AsA was transported from mature leaves through the phloem and transported to root tips, shoots and floral organs with an up to 10-fold higher biosynthetic capacity in source tissues. Assuming that the same processes occur in rice, genotypes with lower capacity to synthesize AsA in the shoot, such as IR74 and ND6172 would consequently also provide less AsA to the roots when needed for the detoxification of ROS under low Zn stress. These considerations point out the necessity to further investigate AsA transport from source to sink tissues and across sub-cellular compartments in plants, which remains a poorly understood phenomenon.

Redox processes and AsA level play important roles not only in stress response but also in plant development. Several studies reported interactions between cell development, plant growth and AsA concentration (Conklin, 2001; del Carmen Córdoba-Pedregosa et al., 2003). Thus the ability of RIL46 to maintain root growth under stress conditions (Table 1) could be related to an almost 50 % higher AsA level than in IR74.

In conclusion, Zn deficiency leads to enhanced ROS formation in both shoot and roots of rice plants, which occurred prior to the formation of visible symptoms. Pairwise genotype comparison suggested that AsA level was important in maintaining cellular redox homeostasis and avoiding oxidative stress under Zn deficiency. The data clearly illustrate the importance of redox homeostasis for the development of rice varieties adapted to Zn deficient soils.

Acknowledgements

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Chapter 3: Ascorbate biosynthesis in rice (Oryza sativa L.) and its involvement in stress tolerance and plant development

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3.1 Abstract

Ascorbic acid (AsA) biosynthesis and its implications for stress tolerance and plant development were investigated in a set of rice knock-out (KO) mutants for AsA biosynthetic genes and their wild-types. KO of two isoforms of GDP-D-mannose epimerase (*OsGME*) reduced the foliar AsA level by 20-30 %, and KO of GDP-L-galactose phosphorylase (*OsGGP*) by 80 %, while KO of *myo*-inositol oxygenase (*OsMIOX*) did not affect foliar AsA levels. AsA concentration was negatively correlated with lipid peroxidation in foliar tissue under ozone stress and zinc deficiency, but did not affect the sensitivity to iron toxicity. Lack of AsA reduced the photosynthetic efficiency as represented by the maximum carboxylation rate of Rubisco (V_{max}), the maximum electron transport rate (J_{max}) and the chlorophyll fluorescence parameter Φ_{PSII}. Mutants showed lower biomass production than their wild-types, especially when *OsGGP* was lacking (around 80 % reductions). All plants except for KO mutants of *OsGGP* showed distinct peaks in foliar AsA concentrations during the growth, which were consistent with up-regulation of *OsGGP*, suggesting that *OsGGP* plays a pivotal role in regulating foliar AsA levels during different growth stages. In conclusion, our data demonstrate multiple roles of AsA in stress tolerance and development of rice.

3.2 Introduction

Ascorbic acid (AsA) is an important plant metabolite with multiple functions. Certain organs of higher plants contain relatively high concentrations of AsA, which constantly increased during the evolution of plants in line with an expansion of the functions in the plant metabolism (Gest et al. 2013). AsA is primarily considered as an antioxidant (Smirnoff and Pallanca 1996), due to its ability to scavenge reactive oxygen species (ROS) through enzymes of the ascorbate-glutathione cycle involving the enzyme ascorbate peroxidase, or non-enzymatically by reducing H_2O_2 directly to water (Noctor and Foyer 1998). ROS such as superoxide ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$) or hydrogen peroxide (H_2O_2) are constantly produced in plant cells as byproducts of metabolic processes such as photosynthesis (Foyer et al. 1994), but different biotic and abiotic stresses can cause an excess production of ROS leading to protein damage, lipid peroxidation and finally cell death. Besides its role as an antioxidant, AsA is a co-factor of many metal-containing enzymes and therefore influences important enzymatic reactions by keeping the transition metal ion center in its reduced form (Davey et al. 2000). AsA performs different functions during photosynthesis, predominantly as an antioxidant in chloroplasts to scavenge H_2O_2, but can also act as an alternative electron donor for photosystem (PS) I and PSII (Mano et al. 2004). Cell growth is known to be influenced by AsA through different mechanisms (Córdoba and González-Reyes 1994), for instance cell wall composition, due to the fact that the first intermediates of AsA biosynthesis also function as cell wall components (Ishikawa et al. 2006). Moreover, previous studies reported that AsA influenced the onset of flowering and senescence in *Arabidopsis thaliana* (Conklin and Barth 2004).

Different AsA biosynthetic pathways in plants have been proposed. The predominant biosynthetic pathway uses glucose-6-phosphate as a precursor and proceeds via GDP-D-mannose and L-galactose (Wheeler et al. 1998) mediated by multiple enzymatic steps as reviewed by Ishikawa et al. (2006). Evidence suggests that the major rate limiting steps in this pathway are GDP-mannose-3,5-epimerase (*GME*) and the subsequent step GDP-L-galactose phosphorylase (*GGP*, also known as VTC2), which was shown, for example in tomato (Gilbert et al. 2009). GME converts GDP-D-mannose into both L-galactose and L-gulose, both of which can act as AsA precursors (Major et al. 2005; Wolucka and Van Montagu 2003). Wolucka and Van Montagu (2003) showed that *GME* was partially the target of feedback inhibition, suggesting that it has a regulatory role in AsA biosynthesis. GGP represents the

first step in the pathway committed exclusively to AsA biosynthesis, while the downstream steps are additionally involved in biosynthesis of cell wall components. GGP is so far the only enzyme, which is light-regulated (Müller-Moulé 2008) and possibly regulated by the circadian clock (Dowdle et al. 2008).

Besides the predominant D-mannose/L-galactose pathway, different alternative biosynthesis pathways have been proposed, one of which uses *myo*-inositol as a substrate for the enzyme *myo*-inositol oxygenase (MIOX). However, there is no consensus about whether this pathway substantially contributes to the physiological AsA level in plants (Endres and Tenhaken 2009; Lorence et al. 2004). Another alternative pathway includes D-galacturonic acid as a precursor and proceeds via L-galactonic acid and finally L-galactono-1,4-lactone. This pathway has been shown to occur in strawberry fruits (Agius et al. 2003), but there is no evidence regarding its contribution to the AsA pool in rice (Jo and Hyun 2011).

Since most investigations of AsA biosynthesis focused on the dicot model plant *A. thaliana*, far less is known about AsA biosynthesis in monocots, and its involvement in plant development and stress tolerance. Rice (*Oryza sativa* L.) is an ideal species for such investigations, as it combines the advantages of a model plant with available genome sequences and mutant collections with the fact that it is one of the globally most important agricultural crops. The dynamics of AsA levels throughout development in rice seem to differ from those observed in other species. In maize (*Zea mays*), tobacco (*Nicotiana tabacum*) (Chen et al. 2003) and *A. thaliana* (Zhang et al. 2009), AsA decreased gradually as the tissue matured, whereas AsA levels increased in different rice cultivars until entering the reproductive stage (Lisko et al. 2013), suggesting that AsA might affect flowering and reproductive development. The rice genome contains one or more orthologs of all AsA biosynthetic genes described in *A. thaliana* (Höller et al. 2014a). Our own previous investigations showed that knock-out of one of the rice isoforms of *GME* led to a 20-30 % decrease of the AsA pool in rice, and to increased sensitivity against ozone stress (Frei et al. 2012) and zinc (Zn) deficiency (Höller et al. 2014b). This suggests that the D-mannose/L-galactose pathway plays an important role in AsA biosynthesis in rice as in *A. thaliana*. Other rice AsA biosynthetic genes or putative pathways have not been characterized yet. However, it is not clear yet how cellular AsA concentrations are controlled especially under stress conditions. Manipulation of biosynthesis genes in different species had differential effects (Alhagdow et al. 2007; Badejo et al. 2008; Bartoli et al. 2005; Gilbert et al. 2009) and it was

found only recently that AsA biosynthesis is controlled by feedback regulation in *Arabidopsis* (Laing et al. 2015). However, detailed information about AsA biosynthesis in rice is missing. The homologous overexpression of the recycling enzyme dehydroascorbate reductase (DHAR) in rice lead to enhanced foliar AsA redox state and improved grain yield and biomass under field conditions (Kim et al. 2013). Together, these investigations suggest that the understanding of AsA biosynthesis in rice has important implications in the breeding for biomass and stress related traits of the world's most important food crop.

The aims of this study were (i) to investigate AsA biosynthetic pathways in rice. This was done by characterizing knock-out mutants for putative key genes involved in the D-mannose/L-galactose pathway (*GME* and *GGP*), and the *myo*-inositol pathway (*MIOX*) regarding the levels of foliar AsA, its precursors and degradation products, and the activity of AsA related enzymes. (ii) To test the involvement of AsA in stress tolerance of rice. This was performed by exposing a collection of rice mutants differing in AsA levels to abiotic stresses, which are relevant for global rice production, including high ozone levels, Zn deficiency, and iron toxicity. (iii) To test the involvement of AsA in rice growth, development, and biomass production. In season-long experiments, we monitored AsA levels in different tissues, and measured photosynthesis as well as biomass-related traits of mutants and wild-types.

3.3 Materials and methods

Plant material

Seeds of the T-DNA insertion lines, 3D-00300 (gene Os10g0417600, *OsGME1*, 2A-50129 (gene Os11g0591100, *OsGME2*), 3A-14221 (gene Os12g0190000, *OsGGP*) and 3A-00389 (gene Os06g0561000, *OsMIOX*) were obtained from the Department of Life Science and National Research Laboratory of Plant Functional Genomics, Pohang University of Science and Technology (POSTECH), Republic of Korea (Jeong et al. 2006). These loss-of-function mutants are hereafter denoted as *osgme1-1*, *osgme2, osggp* and *osmiox*. The TOS17 insertion line ND6172 (gene Os10g0417600, *OsGME1,* mutant line hereafter denoted as *osgme1-2*) was previously described (Frei et al. 2012) and was originally obtained from the Rice Genome Center of the National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan (Hirochika 2001; Hirochika et al. 1996). T-DNA or TOS17 insertions were located in the exons or the promoter region of the respective genes (Supplementary Fig. S2). The wild-type background was Dongjin (DJ) for the T-DNA insertion lines and Nipponbare (NB) for the TOS17 insertion line. Homozygous plants were identified from T1 transgenic seeds by triple primer PCR using a combination of gene specific primers and a T-DNA right border primer (Supplementary Table S1). Homozygous T2 seeds were used for the experiments and compared to segregating wild-types. In the case of *osggp* also heterozygous T2 seeds were used.

Experiment 1: Stress treatments

In all experiments, seeds were germinated on netted styrofoam sheets floating on 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$. For the line *osggp* exogenous AsA was provided to the medium at a concentration of 1 mM AsA to increase the survival rate. The seedlings were then transferred to hydroponic tanks filled with nutrient solution with the following composition (Yoshida et al. 1971). The full strength nutrient solution was composed of 2.86 mM N (as NH_4NO_3) , 0.32 mM P (as NaH_2PO_4), 1.02 mM K (as K_2O_4), 1 mM Ca (as $CaCl_2$), 1.65 mM Mg (as $MgSO_4$), 9 µM Mn (as $MnCl_2$), 1 µM Mo (as $(NH_4)_6$ x Mo_7O_{24}), 18.5 µM B (as H_3BO_3), 0.16 µM Cu (as $CuSO_4$ $CuSO_4$), 35.6 µM Fe (as $FeCl_3$), 1 µM Zn (as $ZnSO_4$). The pH was adjusted to 5.5 every other day, and solutions were completely exchanged after 10 days the latest. All experiments were conducted in a glasshouse with nighttime/daytime temperatures of

22°C/28°C and average relative air humidity was 50-63 %. Plants were grown under supplementary artificial lighting to ensure a minimum PPFD of 300 µmol m^{-2} sec^{-1} from 7 am to 8 pm. At least 4 replicates per genotype and treatment were used in all experiments. Shoot samples were harvested, shock-frozen in liquid nitrogen and stored at -80 °C.

***Zn deficiency treatment*:** After 15 days, seedlings were transferred to 60 l hydroponics tanks containing half strength nutrient solution containing no Zn. After six days the treatments were started with full-strength nutrient solution containing either no Zn (-Zn) or 1 µM Zn (+Zn). The nutrient solution was completely exchanged after ten days and samples were harvested after 17 days of treatment.

***Ozone treatment*:** eleven days after germination, seedlings were transferred to 24 l hydroponic tanks containing half-strength nutrient solution, which was exchanged for full-strength nutrient solution after further 7 days. Ozone fumigation was initiated 24 days after the germination at a target concentration of 100 ppb from 09:00 am to 04:00 pm for 6 days (average recorded daytime ozone concentration: 106 ppb). Ozone was produced with a custom-made ozone generator (UB01, Gemke Technik, Ennepetal, Germany) and the concentration was monitored by an ozone sensor (GE 703 O3, Dr. A. Kuntze GmbH, Meerbusch, Germany) attached to the generator and simultaneously with a handheld type ozone monitor (Series 500, Aeroqual, Auckland, New Zealand) (Ueda et al., 2014). Ozone was distributed with an air blower through perforated tubes running above two independent open top chambers (1.3 m width x 2.0 m length x 1.3 m height), each containing one 24 l hydroponic tank with two genotype replicates. Two identical open top chambers with one 24 l tank, respectively, were used as controls without any ozone fumigation (average recorded daytime ozone concentration: 27 ppb).

***Iron treatment*:** 11 days after germination, seedlings were transferred to 60 l hydroponic tanks filled with half-strength nutrient solution, which was exchanged for full-strength nutrient solution 7 days later. Iron treatment was started when plants were 27 days old with 1000 mg l^{-1} Fe^{2+} (as $FeSO_4$ x 7 H_2O) and it lasted for 4 days. Solutions were percolated with N_2 gas for 15 minutes every two hours to prevent Fe^{2+} from re-oxidation and precipitation (Wu et al. 2014).

Experiment 2: Influence of AsA levels on development and yield

(I) Seeds of the mutant lines were germinated as described in experiment 1 and transferred to soil (a German luvisol: 16 % clay, 77 % silt, 7 % sand, 1.2 % organic carbon, pH 6.3) (Schneider 2005) after 12 days. During the first three weeks one pot (1.45 l) contained 3 plants and after three weeks plants were separated into single pots. In total, eight replicates per line were planted, four of which were used for AsA sampling while the others were used for determination of yield parameters. Soil was fertilized 22 days after transplanting (DAT) with 1 g urea, 0.5 g P_2O_5 and 0.5 g K_2O per pot. A second dose of urea (1g per pot) was applied shortly before plants entered the reproductive stage. Plants were grown in a temperature-controlled glasshouse with minimum nighttime/daytime temperatures of 22°C/28°C. Average relative air humidity was 50 %. Shoot samples for AsA measurements were taken at different developmental stages (0, 7, 21, 35, 54, 61, and 76 DAT) always at the same time (10 am). From 0 DAT to 21 DAT, samples from three plants were pooled into one sample. From 35 DAT the second youngest fully expanded leaf of individual plants was harvested as one sample. On 61 and 76 DAT, ten seeds per plant were harvested for AsA measurement.

(II) Because of the very poor growth and limited seed production of the *osggp* plants, a separate experiment was conducted using hetero- and homozygous *osggp* plants and their segregating wild-type. In total 100 seeds obtained from a T2 heterozygous plant were germinated on netted styrofoam sheets. All seedlings (including those that did not germinate) were genotyped and the germination rate was recorded. After 14 days, six replicates of homozygous, heterozygous and wild-type plants were transferred to soil as described above with three plants per pot (1.45 l) and genotyped. Two weeks later, plants were fertilized as described above. Additionally, 1 g urea per pot was again applied on 70 DAT. Plants were grown in a temperature-controlled glasshouse with minimum nighttime/daytime temperatures of 22 °C/28 °C. Average relative humidity was 55 %. Since this experiment was conducted during spring/summer time with increasing day-length, the growing season was longer than that of the above experiment. Therefore shoot samples were taken at 0, 14, 35, 56, 77, 98 and 119 DAT, which represented the same growth stages as in the previous experiment. From 0 DAT to 35 DAT, three plants were pooled as one sample. Afterwards, the second youngest fully expanded leaf was harvested. On the last two sampling days, ten seeds per plant were harvested to determine AsA levels. To obtain further yield data the experiment was repeated as described above. In total, six wild-type plants, 21 heterozygous and five homozygous

osggp plants were grown until maturity and dried for 3 days at 50 °C to measure biomass production.

Biochemical analyses

Reduced and oxidized AsA was measured in shoots according to Ueda et al. (2013) immediately after harvesting. Shoot and leaf material was ground in liquid nitrogen and about 80 mg were dissolved in 1 ml of 6 % metaphosphoric acid (MPA) and 1 mM ethylenediaminetetraacetic acid (EDTA). Samples were then centrifuged for 20 min at 15 000 g and 4°C, and the supernatants were used for further analyzes. Ten µl of the extract were added to the reaction mix of 80 mM potassium phosphate buffer (pH 7.0) and 0.1 U ascorbate oxidase (AO) for the measurement of reduced AsA. In the case of oxidized AsA, 10 µl of extract were added to 80 mM potassium phosphate buffer (pH 7.8) and 4 mM dithiothreitol (DTT). A blank well was added for every sample where the amount of AO or DTT was substituted by the same amount of 80 mM potassium phosphate buffer, pH 7 or pH 7.8, respectively. Absorbance was monitored in a microplate reader (Powerwave XSII, BioTek) at 265 nm in UV-transparent 96-well microplates until it was constant (ε = 14.3 mM^{-1} cm^{-1}). Total AsA was calculated as the sum of reduced and oxidized AsA.

Malondialdehyde (MDA) was measured according to Hodges et al. (1999). Approximately 100 mg of shoot or leaf material was dissolved in 1 ml 0.1 % trichloroacetic acid (TCA) and centrifuged at 14000 g at 4°C for 15 min. The extraction step was repeated once and supernatants were combined. 250 µl of plant extract was added to the same amount of 0.01 % (v/v) 2,6-di-tert-butyl-4-methylphenol (BHT) in 20 % TCA (v/v) and another 250 µl of plant extract was added to 0.01 % (v/v) BHT and 0.65 % 2-thiobarbituric acid (TBA) in 20 % TCA (v/v). Samples were incubated at 95°C for 30 minutes and incubated on ice for 5 minutes afterwards. After centrifugation at 8000 g for 10 minutes at 4°C, absorbance was measured at 440, 532 and 600 nm with a microplate reader (Powerwave XSII, BioTek).

Enzyme analyses

Enzyme activities were monitored in a microplate reader (Powerwave XSII, BioTek) using 96-well microplates, except for APX, which was measured in 1 ml cuvettes as described by Frei et al. (2010a). Enzyme activities of APX, MDHAR and GR were measured using the same plant extract with four experimental and two analytical replicates. Around 100 mg of flash frozen and ground leaf material was dissolved in 1.5 ml of 50 mM potassium phosphate

buffer (pH 7.8) containing 1 mM AsA and 1 mM EDTA, vortexed, and centrifuged for 30 min at 10000 g and 4°C.

The reaction mix for APX activity (1 ml) contained 100 mM potassium phosphate buffer (pH 6.8), 0.6 mM AsA, 10 µl of 0.03 % H_2O_2, and 100 µl of enzyme extract. The oxidation of AsA was monitored at 290 nm for 30 sec ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

To determine MDHAR activity the reaction mix (100 µl) contained 50 mM TRIS-HCl buffer (pH 7.6), 0.1 mM NADH, 2.5 mM AsA, 0.1 U ascorbate oxidase and 10 µl of the plant extract. The oxidation of NADH was monitored at 340 nm for 3 minutes ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

GR activity was measured in a reaction mix (100 µl) containing 50 mM potassium phosphate buffer (pH 7.8), 0.12 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 10 µl of plant extract. Oxidation of NADPH was monitored at 340 nm for 3 minutes ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

DHAR activity was determined according to Hossain and Asada (1984). Approximately 100 mg of flash-frozen leaf material was ground and dissolved in 1.5 ml of 50 mM TRIS-HCl, 100 mM NaCl, 2 mM EDTA and 1 mM $MgCl_2$. Samples were centrifuged at 13000 g for 5 minutes at 4 °C. The reaction mix (100 µl) contained 50 mM potassium phosphate buffer (pH 6.5), 0.5 mM DHA, 5 mM reduced glutathione (GSH) and 10 µl of plant extract. Absorbance was followed at 265 nm for 3 minutes ($\varepsilon = 14\ mM^{-1}\ cm^{-1}$).

Metabolomic analyses

Metabolomic analyses were carried out as described in Höller et al. (2014a). In brief, soluble sugars were measured enzymatically using an microplate reader (Synergy HT, Tecan, Crailsheim, Germany) with plant extracts derived from ethanol (80 %) extraction. Sugar alcohols were analyzed using an ion chromatography system (Dionex, Idstein, Germany) and separated using an anion-exchange column system. Amino acids were separated by ethanol (80 %) and derivatized using a fluorescing reagent AQC (6-aminoquinolyl-N-hydroxysuccinimidylcarbamat). Amino acid concentrations were determined using a reversed phase Alliance HPLC system (Waters Company) connected to a fluorescence detector (300 nm excitation wavelength, and 400 nm emission wavelength). The concentration of all other metabolites were analyzed using a Dionex ICS 5000 (Dionex) coupled to a mass spectrometer, Agilent 6490 triple Quad as essentially described in Höller et al. (2014a).

RT-PCR

Samples were immediately frozen in liquid nitrogen and ground to a fine powder. RNA was extracted using the peqGOLD Plant RNA Kit (Peqlab, Erlangen, Germany). Residual DNA was removed using an RNase-free DNase (Promega, Mannheim, Germany) and RNA concentration was determined using a Nanodrop2000C spectrometer (Thermo Scientific, Schwerte, Germany). Six hundred ng of total RNA was reverse transcribed with the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany). Thereafter 30 ng of cDNA were used as a template for PCR using gene specific primers (Supplementary Table S1) and the following conditions: An initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C, extension at 72 °C for 45 s. Primer efficiency was tested by serial dilutions of cDNA templates.

Midday photosynthetic measurements

Midday ambient carbon assimilation rate (A), stomatal conductance (g), chlorophyll fluorescence parameters and A-Ci curves were measured on the second youngest fully expanded leaf of each plant between 10:00 and 14:00 with four experimental replicates (Chen et al. 2011). Measurements were carried out with a portable photosynthetic gas exchange system (Li-Cor 6400 portable photosynthetic system, LI-COR, Inc., Lincoln, Nebraska, USA) with a leaf fluorescence chamber head. Leaves were measured under a PPFD of 700 µmol m^{-2} s^{-1} and a CO_2 reference value of 400 ppm with a leaf temperature of 28°C. Chlorophyll fluorescence parameters were taken after saturating flashes once the raw fluorescence value has stabilized. The operating quantum efficiency of photosystem II (φPSII) was calculated from the equation $F_q`/F_m`$, where $F_m`$ is the maximum fluorescence under actinic light and $F_q`$ is the difference between $F_m`$ and F` (level of fluorescence before the saturating flash). Photochemical quenching (q_P), which includes photosynthesis and photorespiration, can be calculated from the equation $F_q` / F_v`$ where $F_v`$ is the difference between $F_m`$ and $F_0`$, the minimal fluorescence under actinic light. NPQ such as heat dissipation can be calculated from $NPQ = F_m-F_m`/ F_m`$, where F_m is the maximum fluorescence after a saturating flash.

For CO_2 response curves A was measured under different CO_2 concentrations ($[CO_2]$) as follows: 400, 200, 100, 50, 400, 500, 600, 700, 800, 1000 mmol mol^{-1} (Chen et al. 2011). To solve the maximum carboxylation rate of ribulose 1,5-bisphosphate carboxylase/oxygenase

(Rubisco) ($V_{c,\ max}$) and the maximum electron transport rate (J_{max}) the A-Ci curve fitting model of Sharkey et al. (2007) was used.

3.4 Results

AsA metabolism

In contrast to wild-type plants, the homozygous mutant plants *osgme1-1*, *osgme1-2*, *osgme2*, *osggp* and *osmiox* did not show any mRNA expression of the targeted genes (Fig. 1a). All mutants except *osmiox* showed significant decreases in total AsA compared to their wild-types (Fig. 1b). The three mutant lines with a knock-out in either *OsGME1* or *OsGME2* showed a similar reduction of AsA levels in a range of 20 to 30 %, compared to the corresponding wild-type, while *osggp* mutants displayed the most drastic reduction of total AsA concentration (around 80 %). As no reduction in AsA levels was seen in the *osmiox* line, we concluded that this gene may not contribute substantially to AsA biosynthesis in rice and excluded the mutant from further analyses.

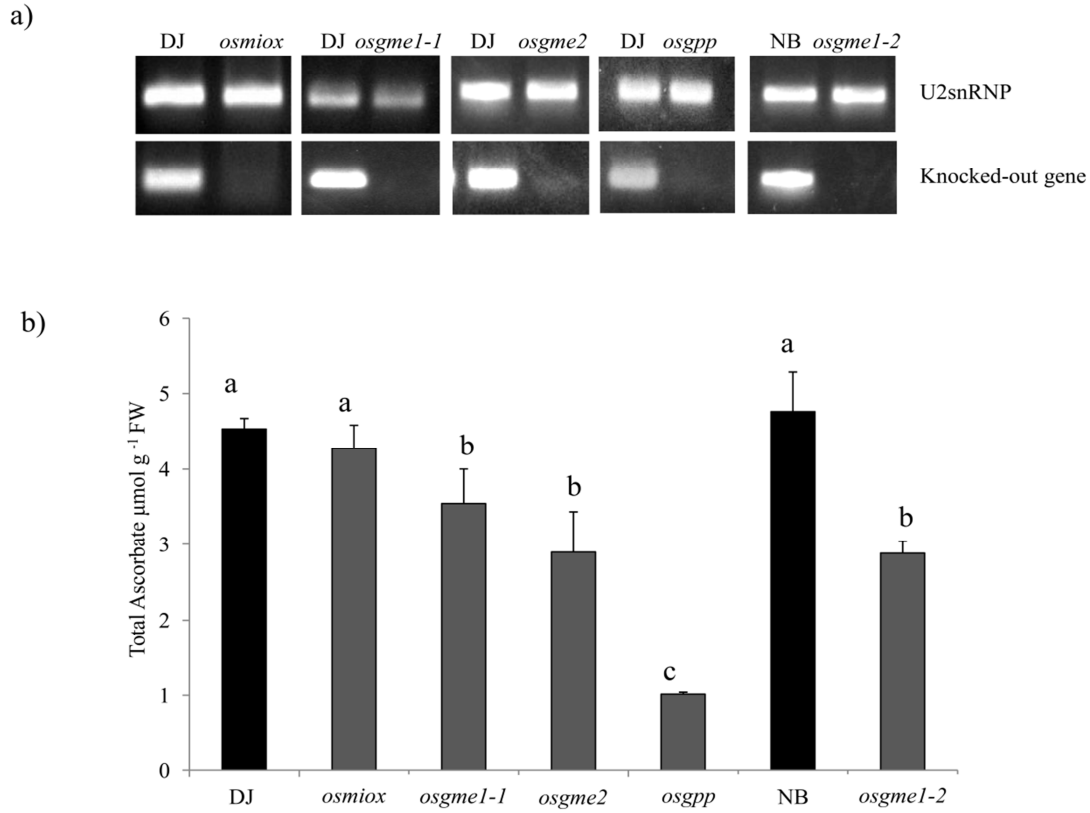


Fig. 1: a) Expression of the genes *OsMIOX*, *OsGME1-1*, *OsGME2*, *OsGGP* and *OsGME1-2* in their corresponding mutant lines and their wild-types (DJ and NB). RT-PCR was performed on mRNA extracted from three weeks old shoot tissue. U2snRNP was used as a reference gene.b) Total ascorbate concentrations in shoots of the different mutant lines (grey bars) and their corresponding wild-types (black bars). Data bars not sharing the same superscript letter are statistically different at $P < 0.05$ by LSD-test. Vertical bars indicate standard errors (n=6).

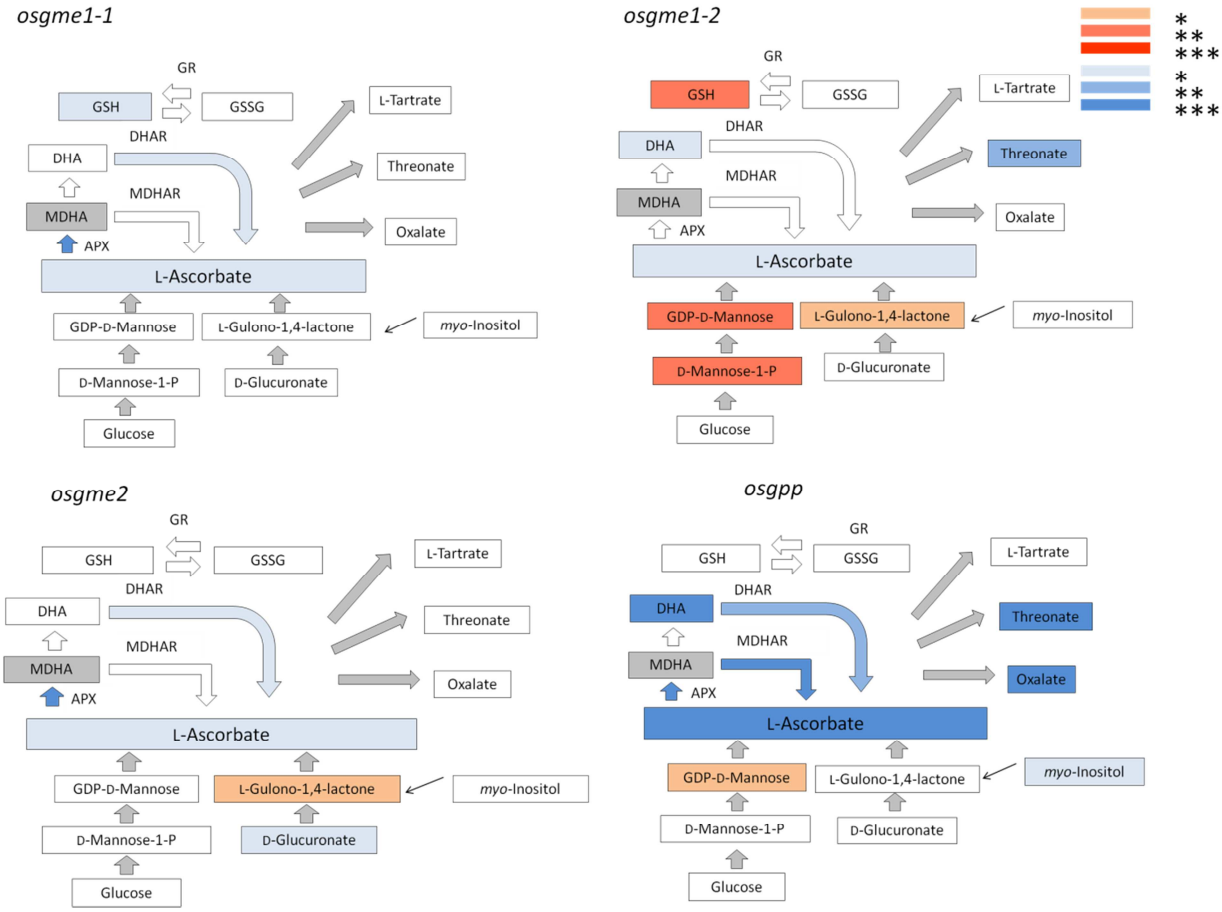


Fig. 2: Effect of a knock-out in ascorbate biosynthesis genes on ascorbate metabolism. Boxes indicate concentrations of ascorbate precursors, recycling or degradation products, arrows indicate enzyme activity. Red or blue color indicate significant up- or down-regulation compared to the wild-type, while color indicates no significant difference and grey color indicates not determined. Significant differences were determined by LSD-test with the following significance levels: 0.05: light red/blue, 0.01: medium red/blue, 0.001: dark red/blue (n=4).

We then analyzed how changes in AsA levels caused by impaired biosynthesis affected AsA metabolism as represented by the levels of AsA precursors, degradation products, and the activity of the enzymes of the AsA-glutathione cycle. AsA precursors tended to accumulate in the mutant lines, which lead to significantly higher substrate concentrations up-stream of the lost enzyme function in the *osgme1-2* and *osggp* mutants (Fig. 2). Moreover, significant increases in the concentration of L-gulono-1,4-lactone, an alternative product of the epimerization of GDP-D-mannose by GME, were observed in two lines (*osgme1-2* and *osgme2*). The activities of the enzymes APX and DHAR were significantly down-regulated in

three out of four mutant lines, respectively. Glutathione levels were affected in the *osgme1-1* and *osgme1-2* knock-out lines in an inconsistent way, which could be related to their different genetic backgrounds (DJ versus NB). The concentration of putative AsA degradation products was significantly lower in the *osggp* mutant (threonate and oxalate), and in the *osgme1-2* mutant (only threonate) (Fig. 2). Generally, the strongest response of the AsA metabolism was observed in the *osggp* mutant, which could be expected considering its substantial decline in AsA level by around 80 %. A number of further metabolites not directly related to the AsA metabolism were also measured (Supplementary Table S2), since we found significant metabolomic changes like increased trehalose and proline levels of rice plants in response to Zn deficiency in a previous study (Höller et al. 2014a). However, no consistent changes in concentrations of amino acids, sugars (apart from sugars involved in the AsA metabolism) or organic acids were detected.

To analyze if a knock-out of the *OsGME* genes causes altered expression of the upstream gene *OsGGP*, we measured gene expression of *OsGGP* in all *OsGME* lines (Supplementary Fig. S1). Compared to the wild-type, *OsGGP* expression increased significantly in *osgme1-2* and *osgme2* mutant plants but not in *osgme1-1*. This suggested that loss of either GME isoform is compensated for by an up-regulation of *OsGGP*.

Abiotic stress tolerance

We conducted further experiments to investigate the implications of AsA for the tolerance of rice against three important stresses affecting rice production: Zn deficiency, ozone pollution, and iron toxicity. MDA concentration was used as a metabolic marker for oxidative stress, as it is a product of lipid peroxidation of cell membranes, caused by excessive production of ROS. Interestingly, significant negative correlations between AsA concentration and MDA levels occurred even under control conditions in the three experiments conducted (Fig. 3). However, as opposed to stress treatments, the plants did not show any visible stress symptoms and had a lower MDA level under control conditions, with the exception of the *osggp* mutants (Fig. 3a, Supplementary data), which showed visible symptoms of oxidative stress and high MDA concentrations even under control conditions. In the stress treatments, significant negative correlations between AsA and MDA were observed under Zn deficiency (Fig. 3a) and ozone stress (Fig. 3b), but not under iron toxicity (Fig. 3c).

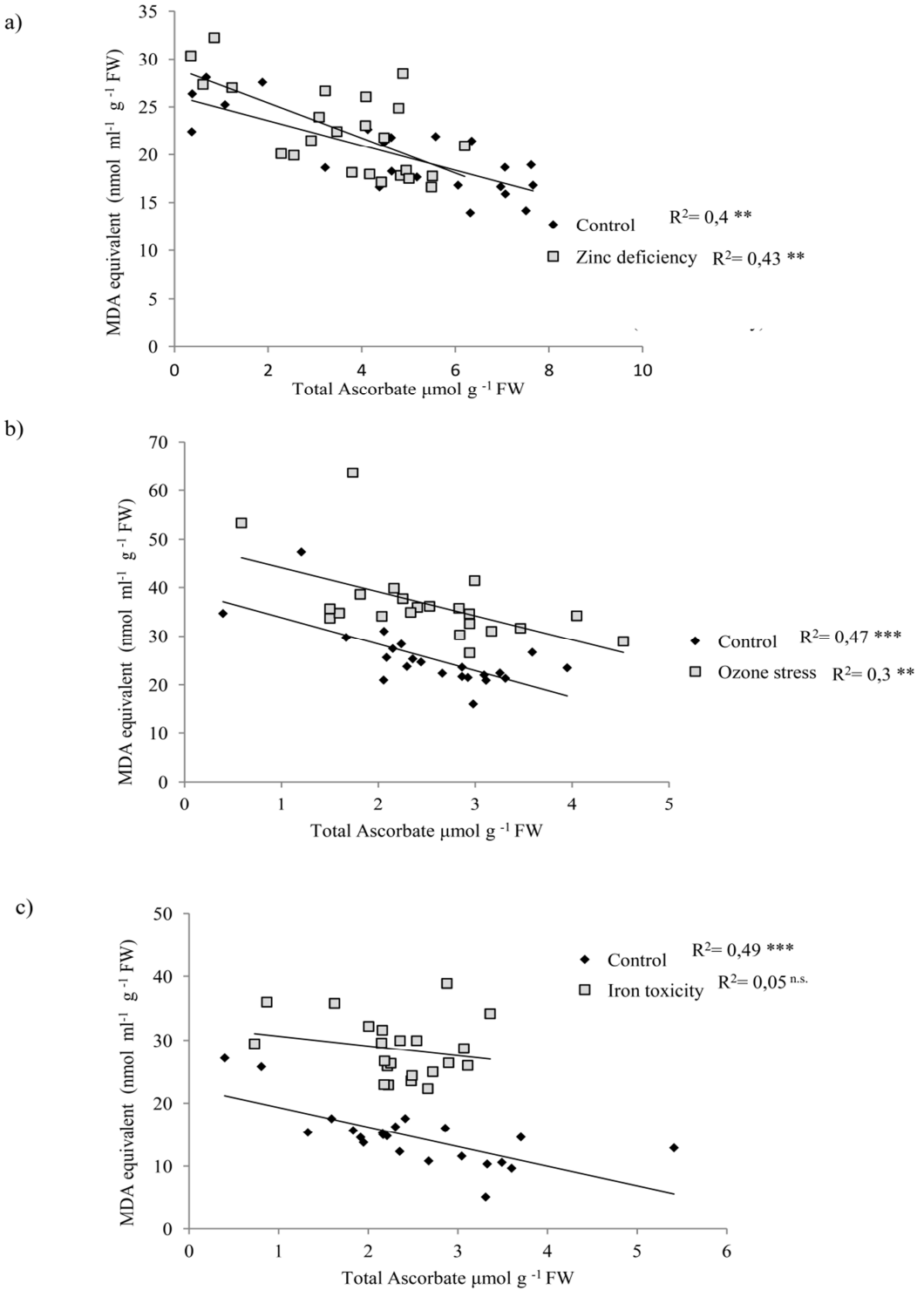


Fig. 3: Linear regression of foliar ascorbate levels versus malondialdehyde concentrations of a set of ascorbate biosynthesis mutants and their wild-types under control conditions (black dots) and different abiotic stress conditions (light dots): zinc deficiency (a), ozone stress (b) and iron toxicity (c). The coefficient of determination (R^2) is shown for every correlation under control and stress conditions. Significance of correlations was determined by a two-sample t-test with the following significance levels *, 0.05, **, 0.01, ***, 0.001 (n=4).

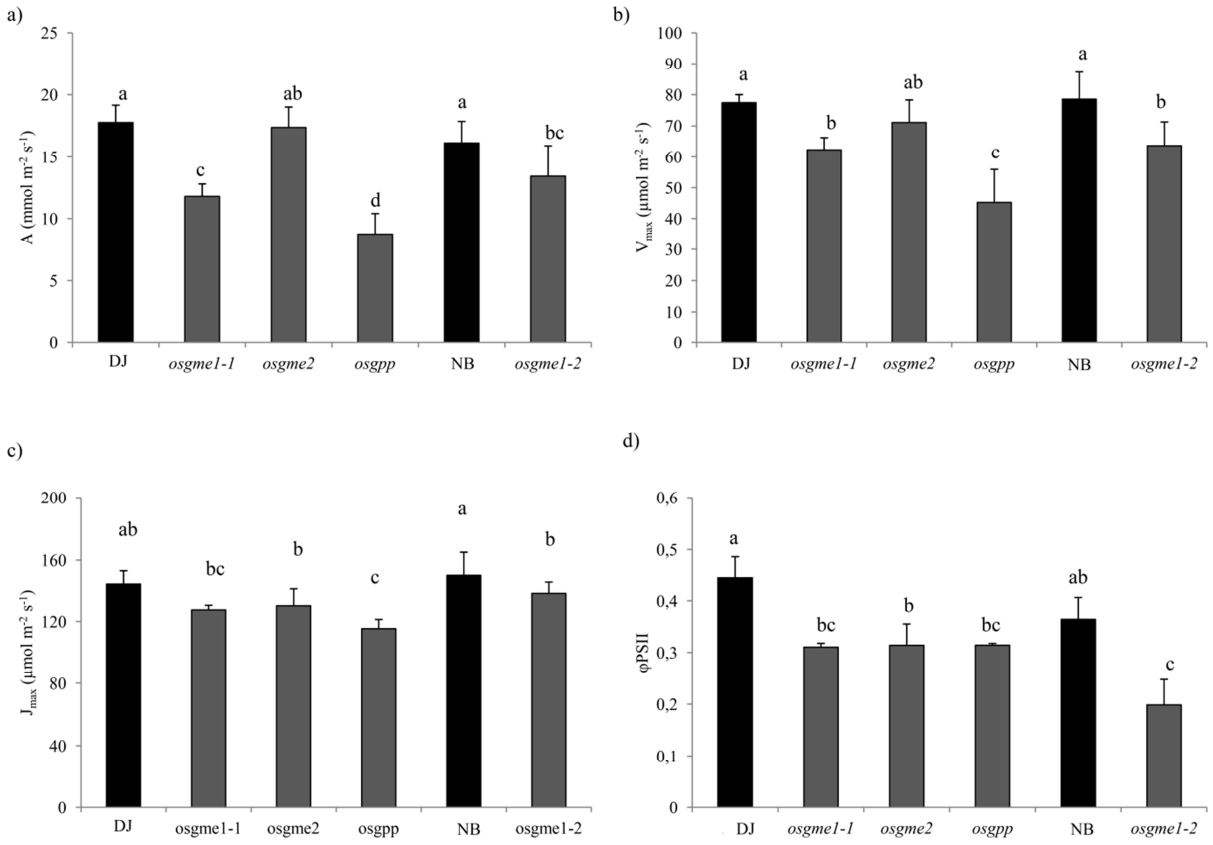


Fig. 4: Photosynthetic parameters of the mutant lines (grey bars) and their corresponding wild-types (black bars) determined by chlorophyll fluorescence analyses and ACi curve measurements: a) Midday ambient carbon assimilation rate (A). b) The maximum carboxylation rate of ribulose 1,5-bisphosphate carboxylase/oxygenase ($V_{c,\ max}$). c) The maximum electron transport rate (J_{max}). d) The operating quantum efficiency of photosystem II (Φ_{PSII}). Measurements were taken on the second youngest fully expanded leaf of each plant between 10:00 and 14:00. Data bars not sharing the same superscript letter are statistically different at $P < 0.05$ by LSD-test. Vertical bars indicate standard errors (n=4).

Photosynthetic capacity

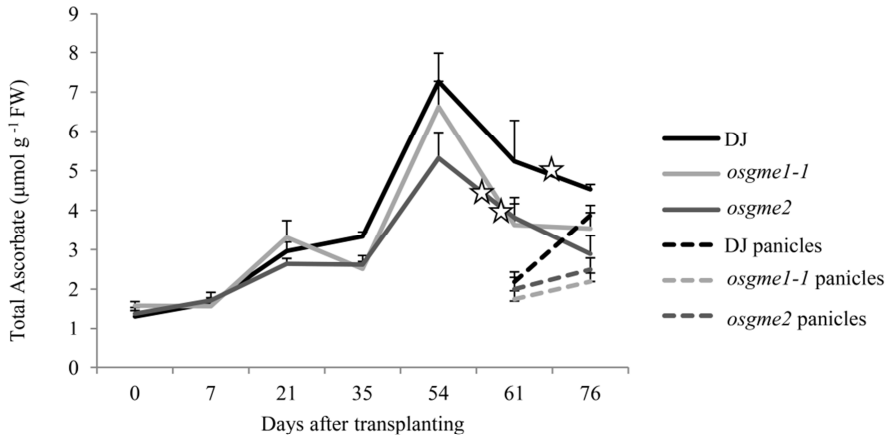
To evaluate the effect of different AsA levels on photosynthetic capacity, we measured different parameters of photosynthesis (Fig. 4). Midday ambient carbon assimilation rate (A) was significantly decreased by 20-50 % in the mutant lines except for *osgme2*, with the strongest reduction occurring in *osggp* (Fig. 4a). Stomatal conductance was not significantly affected in any of the mutant lines (Supplementary Table S2). Biochemical photosynthetic capacity, as represented by V_{max} (the maximum carboxylation rate of Rubisco) was reduced by 20-40 % in all the mutants except for *osgme2* (Fig. 4b). J_{max}, the maximum electron transport rate, was impaired in *osggp* and *osgme1-2* (10-30 %), *i.e.* the mutants with the

lowest foliar AsA concentrations (Fig. 4c). Differences between the mutant lines and their wild-types were also observed in the chlorophyll fluorescence parameters. Φ_{PSII} was reduced by 30-50 % in all mutant lines, indicating that the fraction of absorbed photons used for photochemistry in a light-adapted leaf was lower in plants with decreased AsA levels (Fig. 4d). In conclusion, photosynthetic capacity was significantly impaired by a lower AsA concentration.

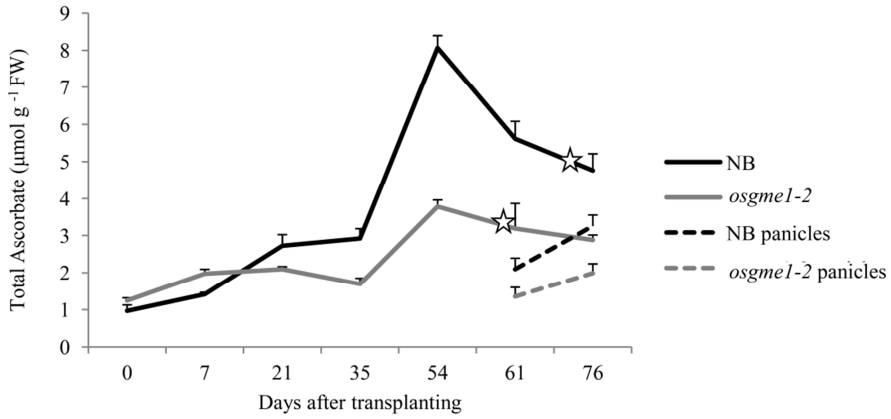
Development and yield

Because AsA has been proposed to influence plant development and the onset of flowering, we conducted a long-term experiment to evaluate changes of AsA levels throughout plant development and the impact of different AsA concentrations on flowering and biomass parameters. A first experiment was conducted with all the mutant lines except for *osggp*. Foliar AsA concentrations peaked twice during development, with a small increase 21 DAT and an increase of almost 50 % at 54 DAT, shortly before the onset of flowering (Fig. 5). The difference in AsA concentrations between the mutants and wild-types was most pronounced during the second peak and thereafter, indicating a role of AsA during the reproductive stage. AsA was additionally measured in the developing and fully developed panicles. Total panicle AsA level was higher in the wild-types than in all the mutant lines, especially in the fully developed panicles. Moreover, panicle AsA level increased on the second sampling day, in parallel to a decrease of foliar leaf AsA level. The majority of AsA in dried seeds was present in its oxidized form and lower in the mutant lines (Supplementary Table S2). The mutant lines started to flower earlier than wild-type plants and total yield decreased significantly. Rough (unmilled) rice yields in *osgme1-1*, *osgme1-2* and *osgme2* were reduced by 56 %, 46 % and 17 %, respectively (Table 1). The proportion of filled grains was more than 90 % in the wild-type plants, while mutant lines only had 50-70 % of filled grains, indicating that a lack of AsA increased spikelet sterility. Thousand kernel weight (TKW) was in tendency lower in the mutant lines, but did not show any significant differences compared to wild-type plants (Table 1).

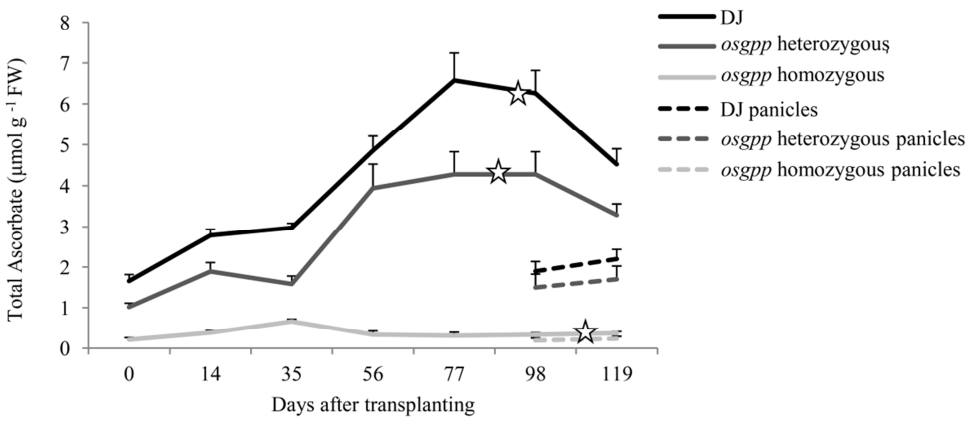
a)


Total Ascorbate (µmol g -1 FW)
Days after transplanting
0
7
21
35
54
61
76
DJ
osgme1-1
osgme2
DJ panicles
osgme1-1 panicles
osgme2 panicles

b)


Total Ascorbate (µmol g -1 FW)
Days after transplanting
0
7
21
35
54
61
76
NB
osgme1-2
NB panicles
osgme1-2 panicles

c)


Total Ascorbate (µmol g -1 FW)
Days after transplanting
0
14
35
56
77
98
119
DJ
osgpp heterozygous
osgpp homozygous
DJ panicles
osgpp heterozygous panicles
osgpp homozygous panicles

d)

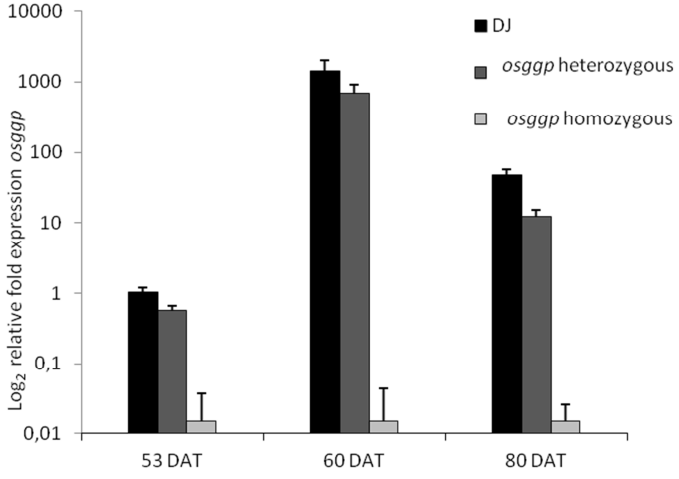

Log2 relative fold expression osggp
10000
1000
100
10
1
0,1
0,01
53 DAT
60 DAT
80 DAT
DJ
osggp heterozygous
osggp homozygous

Fig. 5: Foliar and panicle ascorbate (AsA) concentration throughout the whole growing season on seven sampling days representing different developmental stages (continuous line). Additionally, AsA level was measured in the young and fully developed panicles, respectively (broken line). Asterisks indicate the onset of flowering. a) AsA levels of the mutant lines *osgme1-1* and *osgme-2* and the wildtype Dongjin (DJ). b) AsA levels of the mutant line *osgme1-2* and the wild-type Nipponbare (NB). c) AsA levels of homo-and heterozygous *osggp* mutants and the segregating wild-type Dongjin (DJ). Longer growing season in this experiment was caused by a longer day length compared to a) and b). Vertical bars indicate standard errors (n=4). d) Relative expression of the *OsGGP* gene in homo-and heterozygous *osggp* lines and their wild-types (DJ). Expression levels are expressed as fold-changes relative to the calibrator DJ (wild-type) on the first sampling day. Samples were taken on three sampling days: one week before the onset of flowering (53 DAT), one week after the onset of flowering (60 DAT) and three weeks after the onset of flowering (80 DAT). Data bars not sharing the same superscript letter are statistically different at $P < 0.05$ by LSD-test. Vertical bars indicate standard errors (n=4).

In a second experiment, growth parameters, photosynthetic activity and the AsA level of hetero- and homozygous *osggp* plants and their segregating wild-type were analyzed throughout their whole development. We included heterozygous plants in this experiment, which had around 30 % lower AsA concentrations than the homozygous wild-types. Homozygous mutant lines had a lower germination rate than heterozygous and wild-type plants (Table 2). As in the previous experiment, total AsA levels peaked shortly before the start of flowering, but AsA levels increased more slowly than in the previous experiment, probably due to the longer growing season caused by a longer day length during that experiment (Fig. 5c). Homozygous *osggp* plants had constantly very low AsA concentrations that never exceeded 1 μmol g^{-1} FW. These plants also did not show any seasonal pattern such as increasing AsA levels before entering the reproductive stage. AsA levels in the panicles of heterozygous and wild-type plants increased on the second sampling day with a generally lower concentration in heterozygous plants. As expected, AsA levels in the panicles of homozygous plants were below 0.3 μmol g^{-1} FW and did not increase on the second sampling day (Fig. 5c). To further analyze the role of *osggp* in developmental fluctuations of the AsA level, we measured gene expression levels of the *osggp* gene in heterozygous *osggp* plants and their wild-types. In wild-type plants, expression level of *OsGGP* was approximately 1500-fold higher in wild-type plants at the onset of flowering compared to the level in wild-type plants one week before flowering (Fig. 5d). Heterozygous *osggp* plants increased *OsGGP* transcript levels to approximately half of the level wild-type plants, while homozygous plants showed no measurable *OsGGP* expression (Fig. 5d). Biomass production was significantly reduced in homozygous *osggp* plants compared to heterozygous plants as

well as panicle number and panicle weight (Table 2b). Unfortunately, spikelet sterility data could not be taken in this experiment due to constitutively very high sterility presumably caused by low air humidity in the winter season.

Table 1: Yield parameters of the mutant lines and their corresponding wild-types (DJ and NB) determined from soil-grown plants. Data represent means (standard error) determined from soil-grown plants. DW = Dry weight, TKW = Thousand kernel weight. Parameters not sharing the same superscript letter are statistically different at P < 0.05 by LSD-test (n=4).

Line	DW (g)	Rough rice (g plant^{-1})	TKW (g)	Filled grain (%)
DJ	18,6^{b} (0,65)	15,6^{ab} (0,38)	21,8 (0,25)	93,6^{a}
osgme1-1	15,4^{bc} (1,57)	6,8^{c} (1,45)	20,0 (0,64)	58,4^{b}
osgme2	14,9^{c} (1,65)	12,9^{bc} (1,70)	20,1 (0,48)	50,7^{b}
NB	26,9^{a} (0,95)	18,4^{a} (1,87)	21,8 (0,75)	91,2^{a}
osgme1-2	14,7^{bc} (2,26)	9,9^{c} (2,72)	21,5 (0,29)	66,7^{b}

Table 2: Germination rate and biomass production of homo- and heterozygous *osggp* mutant lines compared to their segregating wild type. Seeds of a T3 *osggp* plant (offspring of a T2 heterozygous plant) were germinated and all seedlings were genotyped (including seeds that did not germinate). The ratio of homo- and heterozygous plants and the segregating wild-types (DJ) as well as the germination rate were recorded. Additionally, yield parameters of the hetero-and homozygous *osggp* plants and their segregating wild-types were determined from soil-grown plants as means (standard error). DW = Dry weight. Parameters not sharing the same superscript letter are statistically different at P < 0.05 by LSD-test (DJ: n=6; heterozygous osggp: n=21; osggp homozygous: n=5).

Line	Total seeds	Seeds germinated	Germination rate (%)	DW (g)	Panicle number	Panicle weight (g)
DJ	24	16	67	26,64 ^{ab} (2,61)	12,50 ^{a} (1,20)	4,50 ^{ab} (0,44)
osggp heterozygous	50	31	62	24,19 ^{a} (3,71)	9,95 ^{a} (1,49)	4,44 ^{a} (0,84)
osggp homozygous	26	10	38	5,51 ^{b} (1,93)	3,40 ^{b} (1,44)	0,78 ^{b} (0,38)

3.5 Discussion

The roles of GME and GGP in the regulation of AsA level metabolism in rice

Suppression of different AsA biosynthesis genes affected AsA levels to a varying extent. Knock-out of different genes of the D-mannose/L-galactose pathway caused a decrease of AsA levels in all mutants. The suppressed transcription of either *OsGME1 or OsGME2* had a similar effect on shoot AsA levels in each of the three *osgme* mutants. The moderate decrease of AsA levels by up to 30 % is most likely due to the fact that only one OsGME isoform remained active, suggesting that OsGME1 and OsGME2 contribute to overall AsA levels in an additive manner (Fig. 1b). In fact, *GME* is considered as an important 'hub' in AsA biosynthesis, as it regulates the genes in AsA biosynthesis, whose products are downstream, as shown in tomato (Gilbert et al. 2009). We were able to confirm a co-regulation of *OsGME* with *OsGGP*, since the expression of the *OsGGP* gene was significantly increased when any of the two *OsGME* homologues was suppressed in its expression (Supplementary Fig. S1). Likewise, RNAi silencing of the two homologues of tomato *GME* caused a decrease of AsA level by 40-60 % (Gilbert et al. 2009). These variations between the different species emphasize the complex regulation of the AsA biosynthesis pathway and the fact that there is no universally model for AsA biosynthesis in different plant species. Knock-out of the *OsGGP* gene had a very strong effect on AsA concentration. Although a second homologue of *OsGGP* exists in rice according to the current gene annotation (RAP ID Os01g0901300), the suppression of just one homologue caused a drastic decrease of AsA level by 80-90 % (Fig. 1b). The outstanding role of *GGP* in AsA biosynthesis has also been documented in other species. Overexpression of the kiwifruit homologue of *GGP* in tobacco leaves led to a 3-fold increase of AsA (Laing et al. 2007). Moreover, *GGP* is light-regulated and the expression of the gene was decreased by AsA supplementation, indicating possible feedback inhibition (Dowdle et al. 2008). These findings demonstrate the great importance of *GGP* in AsA biosynthesis, especially since it represents the first step solely committed to AsA biosynthesis and not to the synthesis of cell wall precursors (Linster and Clarke 2008). Apart from the D-mannose-L-galactose pathway, an alternative AsA biosynthesis pathway via *myo*-inositol pathways has been proposed (Lorence et al. 2004), although this pathway is discussed controversially in the literature (Endres and Tenhaken 2009; Zhang et al. 2008). In the rice *osmiox* mutant we did not observe any measurable difference in foliar AsA levels to wild-type levels, neither under control conditions (Fig. 1b), nor in stress treatments (data not shown).

Therefore, we conclude that at least the specific isoform of *myo*-inositol oxygenase targeted in the *osmiox* mutant (RAP-ID Os06g0561000) does not contribute to AsA biosynthesis in rice.

Regarding the impact of gene knock-outs on AsA metabolism, we observed that precursors in the AsA pathway tended to accumulate in the mutant lines, especially in *osgme1-2*. Intermediates of the predominant D-mannose/L-galactose pathway and the pathway proceeding via L-gulose were affected, indicating an equally important role of both pathways in rice (Fig. 2). The ability of OsGME to catalyze the synthesis of both L-galactose and L-gulose has been shown before (Watanabe et al. 2006). Moreover, the activity of enzymes involved in recycling processes was lower in mutant lines, suggesting that their regulation depends on substrate availability and that the AsA concentration is a limiting factor for the activity of those enzymes which are involved in recycling (Fig. 2). This is in agreement with the findings of Chao et al. (2010), who increased APX activity in leaves of rice seedlings by a pretreatment with AsA or L-galactono-1,4-lactone, the immediate precursor of AsA. Out of three putative degradation products of AsA, only two were decreased in the mutant lines, especially in *osggp*. Thus, our data provide correlative evidence that threonate and oxalate function as catabolic products of AsA in rice. However, in another study transgenic rice plants with decreased AsA levels showed constant oxalate concentrations (Yu et al. 2010), indicating that further research is needed to determine if oxalate is a direct degradation product of AsA. The degradation of AsA to threonate has been shown in different species, like *Pelargonium crispum, Lemon geranium* (Helsper and Loewus 1982; Saito et al. 1997) or *Rosa* (Green and Fry 2005). Our study suggests that threonate is likely to be a product of AsA catabolism in rice, in contrast to L-tartrate, which was not affected in any of the mutant lines (Fig. 2). It is possible that the degradation of AsA to L-tartrate occurs in Vitaceae only, since most of the literature reports evidence for this catabolic pathway in grapes (Saito and Kasai 1984; Williams and Loewus 1978).

The analysis of osgme and osggp mutants reveals a differential role of ascorbate in abiotic stress tolerance

As AsA has been proposed as an important antioxidant conferring tolerance to multiple environmental stresses (Conklin and Barth 2004; Frei et al. 2012; Smirnoff and Pallanca 1996), we conducted a series experiments to test the sensitivity of rice mutants with compromised AsA biosynthesis against Zn deficiency, high ozone stress or iron toxicity. A negative correlation between foliar AsA levels and lipid peroxidation was detected under Zn

deficiency (Fig. 3). This nutrient disorder was proposed to lead to excess ROS production due to the role of Zn in the function of antioxidant-related enzymes such as Cu/Zn superoxide dismutase (Cakmak 2000) or the AsA biosynthetic enzyme phosphomannose isomerase (PMI) (Gracy and Noltmann 1968). Our own previous results demonstrated that the formation of visible symptoms in rice is preceded by a redox imbalance in roots and shoots (Höller et al. 2014b), and that high levels of AsA were correlated with tolerance to Zn deficiency in rice breeding lines (Frei et al. 2010b; Höller et al. 2014a). Together with these previous results, the current data clearly support an important role of AsA in tolerance to Zn deficiency. In the case of ozone stress ROS are produced due to decomposition of ozone in the apoplast, which induces an oxidative burst and cell death via multiple signal transduction pathways (Kangasjarvi et al. 2005). Because AsA is an antioxidant occurring in the apoplast, it has long been suggested as an important protective agent against ozone stress (Conklin and Barth 2004). Our own previous experiments involving the mutant line *osgme1-2* demonstrated that rice yield under ozone stress was decreased by lower foliar AsA levels (Frei et al. 2012). Thus, the current results (Fig. 3) clearly confirm the important role of AsA in preventing oxidative tissue damage under ozone stress. In contrast to these two stress factors, no correlation was seen between AsA levels and lipid peroxidation under iron toxicity (Fig. 3). This nutritional disorder occurs specifically in rice due to excessive uptake of reduced iron (Becker and Asch 2005), which causes production of hydroxyl radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$) (Becana et al. 1998). In this reaction, AsA may play an ambivalent role as an antioxidant. On the one hand it can reduce H_2O_2 to water and thus remove the precursor of the hydroxyl radical, for which no effective scavenger exists in plants (Apel and Hirt 2004). On the other hand, the redox potential of AsA is sufficiently negative to reduce Fe^{III} to Fe^{II} (Buettner and Jurkiewicz 1996), thereby fueling the Fenton reaction and in turn the production of the particularly harmful hydroxyl radical. Thus our results suggest that AsA does not play a positive role in terms of tolerance to ferrous iron toxicity, while it promotes tolerance against ozone stress and Zn deficiency.

The analysis of osgme and osggp mutants reveals a promotive role of AsA in photosynthesis

AsA has many functions in photosynthesis, including it function as an antioxidant to scavenge photo-chemically produced ROS in the chloroplasts (Smirnoff 2000). Additionally, AsA constitutes an electron donor for violaxanthin de-epoxidase, an enzyme involved in the

xanthophyll cycle to dissipate excess excitation energy (non-photochemical quenching) (Müller-Moulé et al. 2002). Moreover, AsA can be photooxidized in PSI and PSII and thus contribute to photosynthetic electron flow as an alternative electron donor (Mano et al. 2004). In line with these reports, our results showed that lack of AsA had an inhibitory effect on various photosynthetic parameters (Fig. 4). The maximum carboxylation rate of Rubisco (V_{max}) was significantly reduced in three of the four mutants, especially in *osggp*, possibly due to higher chloroplastic ROS levels. It has been shown previously in tomato and cucumber that high levels of H_2O_2 correlated with a lower activity of Rubisco due to oxidative modifications of the enzyme (Liu et al. 2008; Zhou et al. 2006). In two of the mutant lines (*osgme1-2* and *osggp*) the maximum electron transport rate was impaired. AsA can influence the photosynthetic electron flow as an alternative electron donor to PSI and PSII (Mano et al. 2004) (Fig. 4c) and reduce photo-damage caused by excess excitation leading to the formation of ROS (Huang et al. 2005). Reduction of the actual quantum yield of PSII (φPSII) in the mutant lines could have been caused by any of these factors. Our result suggests an important role of AsA in maintaining high photosynthetic capacity in rice, which is already advantageous under normal growth conditions, but could be of additional benefit under adverse environmental conditions.

OsGME and OsGGP-dependent AsA levels play a role in the development of rice plants

AsA, or more likely its redox state is important for cellular processes regulating growth, such as cell division and cell cycle activity (Potters et al. 2002). In our study, a lower AsA level affected the growth of several mutant lines significantly (Table 1). An *A. thaliana* loss-of-function mutant for GDP-mannose pyrophosphorylase had a 70 % lower AsA level than wild-type plants and showed a decreased growth rate (Veljovic-Jovanovic et al. 2001). However, this step in AsA biosynthesis is non-specific and additionally involved in cell wall synthesis. In contrast, the *osggp* mutant used in our study showed the most severe growth defects, although GGP is the first enzyme committed only to AsA biosynthesis, suggesting that the internal AsA level directly affected biomass production (Table 2). Decreased photosynthetic capacity (Fig. 4) and the fact that AsA is involved in controlling cell elongation and division (Noctor and Foyer 1998) may be responsible for the decrease in biomass.

In the course of rice development, AsA levels peaked shortly before the onset of flowering (Fig.5 a-c). This pattern is in contrast to those found in other species such as *A. thaliana*, where AsA levels constantly declined during their development (Conklin et al. 1996; Lisko et

al. 2013; Zhang et al. 2009). Although the *osgme* mutant lines had lower AsA levels than the wild-types, a similar seasonal pattern in AsA levels of the shoots was observed. In contrast, homozygous *osggp* plants did not show any distinct seasonal dynamics of foliar AsA levels, suggesting that OsGGP makes an important contribution to the phenological increase of AsA levels. Indeed, at the onset of flowering the *OsGGP* gene was upregulated up to 1500 fold in wild-type plants and up to 700-fold in heterozygous *osggp* plants, which had 30 % lower AsA levels than the wild type. The fact that a major increase in foliar AsA levels occurred just before the initiation of flowering (Fig.5) suggests a physiological role of AsA in the reproductive development. Also, the decline of foliar AsA levels during panicle development was paralleled by an increase in panicle AsA levels, which may suggest transport of AsA from leaves to the reproductive organs. It has been shown for *Vicia faba* that young seeds are not capable of producing AsA and are provided with AsA by the maternal tissues (Arrigoni et al. 1992). Moreover, studies in tomato have shown that AsA can be transported from leaves to fruits (Giovanelli et al. 1999; Massot et al. 2013; Stevens et al. 2007).

All mutants with lower AsA levels relative to their wild-types, except for homozygous *osggp* plants showed an early flowering phenotype, accompanied by decreased yield (except of *osgme2*) and higher seed sterility (Fig. 5, Table 1). In *Arabidopsis thaliana*, mutants with low AsA levels also showed early flowering, probably due to an inhibitory effect on floral induction (Conklin and Barth 2004). However, this was only true for plants grown under long-day conditions, while under short-day conditions plants were delayed in flowering (Veljovic-Jovanovic et al. 2001). Nevertheless, AsA might interact with flowering signals in rice to inhibit early flowering, as it does in *Arabidopsis*, where the expression of LEAFY, a gene involved in floral induction, was reduced by pretreatment with L-galactono-1,4-lactone (Barth et al. 2006). Moreover, ROS and in particular H_2O_2 may interfere with the induction of flowering. *Arabidopsis* plants suffering from increased oxidative stress showed an early flowering phenotype (Lokhande et al. 2003) and in another study an *apx* loss-of-function mutant in *Arabidopsis* flowered earlier than the wild-type (Chai et al. 2014). In the case of homozygous *osggp* the extremely low AsA level and the resulting deterioration of plant fitness might account for the fact that these plants entered the reproductive stage later or not at all in some cases. In these plants, the germination rate was also impaired (Table 2). It has been reported that AsA in dry seeds is only present in its oxidized form (DHA) and that AsA biosynthesis starts 8-20h after germination (Gara et al. 1997). Chen et al. (2014) showed that one of the *Arabidopsis* APX isoforms is important for protecting mature drying seeds from

oxidative stress and to modulate ROS signaling. Tobacco seeds overexpressing APX had enhanced germination rates (Lee et al. 2010) and seed priming with AsA led to enhanced yields in rice (Farooq et al. 2007). The importance of the redox state of the seed for germination was shown by Kranner et al. (2010) in *Pisum sativum*, where oxidative bursts were recorded during germination. However, the production of ROS must be tightly controlled. In this context, antioxidants such as AsA may play an important role in balancing the redox status in germinating seeds.

In conclusion, a decrease of AsA by approximately 25 % already had a negative impact on abiotic stress tolerance in rice (except for iron toxicity). Moreover, this lower AsA levels also reduced photosynthetic capacity, growth and yield. Plants with more severe losses of AsA were hardly able to survive and showed severe growth and developmental defects. This highlights the importance of AsA for maintaining plant fitness even under non-stressed conditions. Especially the severe yield losses caused by a merely moderate reduction of AsA are of great importance, since rice is one of the most important crops in the world. Finally, this study also shows that the D-mannose/L-galactose pathway is the predominant one in rice and that the alternative *myo*-inositol pathway presumably does not contribute to AsA biosynthesis.

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General Discussion

1.1 Outline

The ascorbate (AsA) metabolism was investigated concerning its impact on stress tolerance and plant fitness in rice.
In a first approach, the impact of zinc (Zn) deficiency on the whole AsA metabolism was examined by transcriptional and metabolomic profiling in two genotypes differing in Zn efficiency. The detailed analysis of AsA biosynthesis and catabolism revealed higher substrate availability combined with higher expression levels of several biosynthetic genes as possible factors responsible for maintaining a higher AsA level under Zn deficiency. Additionally, the alternative *myo*-inositol pathway was suggested to contribute to a higher AsA level, especially under stress conditions like Zn deficiency, since the predominant biosynthetic pathway is Zn dependent. No limitation of recycling was found in both genotypes, indicating that an increased catabolism of AsA is not the reason for the loss of AsA in the intolerant genotype.

Zn deficiency leads to an increased production of reactive oxygen species (ROS) (Cakmak and Marschner, 1988; Cakmak and Marschner, 1993), however, the process of oxidative stress formation is very complex. Thus, the impact of AsA on redox imbalances caused by Zn deficiency was investigated in more detail in shoots and roots. Oxidative stress appeared before the formation of visible leaf symptoms, which was demonstrated by the comparison of biochemical stress factors combined with the analysis of hydrogen peroxide (H_2O_2) formation by different staining methods. AsA was able to protect plants by maintaining redox homeostasis in shoots and roots. Although several other root-based tolerance mechanisms against Zn deficiency have been suggested, AsA may represent an additional tolerance factor.

To verify the important role of AsA and to investigate its biosynthesis in more detail, a set of different mutant lines with different AsA levels was used. We have already explored the function of AsA as an antioxidant in preventing oxidative stress under Zn deficiency, thus, we analyzed its impact on other abiotic stresses and on plant fitness in general. It was found that even a moderate decrease of AsA concentration impaired the ability of plants to cope with stresses like Zn deficiency and ozone stress. However, this was not true for iron toxicity,

highlighting the ambiguous role of AsA. Moreover, plants with a lower AsA level were not able to maintain photosynthetic capacity and delivered lower yields, which is particularly important since rice is one of the most important crops in the world. Additionally, this study revealed that the alternative *myo*-inositol pathway does presumably not contribute to the AsA pool in rice.

1.2. Zinc deficiency and its impact on rice cultivation

Rice, wheat and maize are considered to be the most important crops in the world and are produced in large amounts. However, a large proportion of the cultivated wheat and maize is used for feeding of live stocks and thus rice can be considered as the most important crop for human nutrition (Maclean et al., 2002). Due to a growing world population the increase of rice yields is particularly important, since the demand for food is rising while production areas of rice are decreasing. Additionally, high-yielding varieties are needed to reduce food prices and avoid major expansions of crop land. During the Green Revolution, high-yielding crop varieties were developed and food production and yields from the most important crops increased extensively (Evenson and Gollin, 2003) (Fig. 1). As an example, 60-85 % of the increase of rice production in China is caused by an expansion of yields (Defeng, 2000). Therefore it was possible to raise grain yields proportional to increases in population during the last years (Fig. 1), however, the cultivars derived from the Green Revolution have currently reached a yield barrier (Cassman, 1999; Kropff et al., 1994). Thus, a remarkable enhancement of rice yield potential is required to keep pace with the growth of population dependent on rice as a staple food.

Abiotic stresses account for major yield losses which can reach up to 40 % in the case of Zn deficiency (Alloway, 2004). Although Zn deficiency is a well-known phenomenon since decades (Yoshida and Tanaka, 1969) and is now considered to be one of the most widespread nutrient disorders in rice (Neue and Lantin, 1994), only little is known about what exactly causes symptoms like leaf bronzing, stunted growth and finally reduced yields in rice. About 50 % of the global soils used for cereal cultivation are considered to be Zn-deficient, especially in South-East Asia and Africa, where rice accounts for a major part of the daily calorie intake (HarvestZinc Project, http://www.harvestzinc.org). Interestingly, a lot of the newly developed high-yielding crop varieties derived from the Green Revolution suffered

more from acute Zn deficiency than older varieties, which are already adapted to the environment. These traditional cultivars are known to be more Zn efficient but give lower yields (Alloway, 2009). Zn deficiency symptoms are even accelerated by the fact that the high-yielding varieties require more fertilization (especially phosphate fertilizer) resulting in increased pH values and high levels of phosphorous. Both soil factors are known to be associated with Zn deficiency (Wissuwa et al., 2006). In this context, it is urgently needed to overcome these limitations caused by stresses like Zn deficiency and to understand the reasons for tolerance against it.

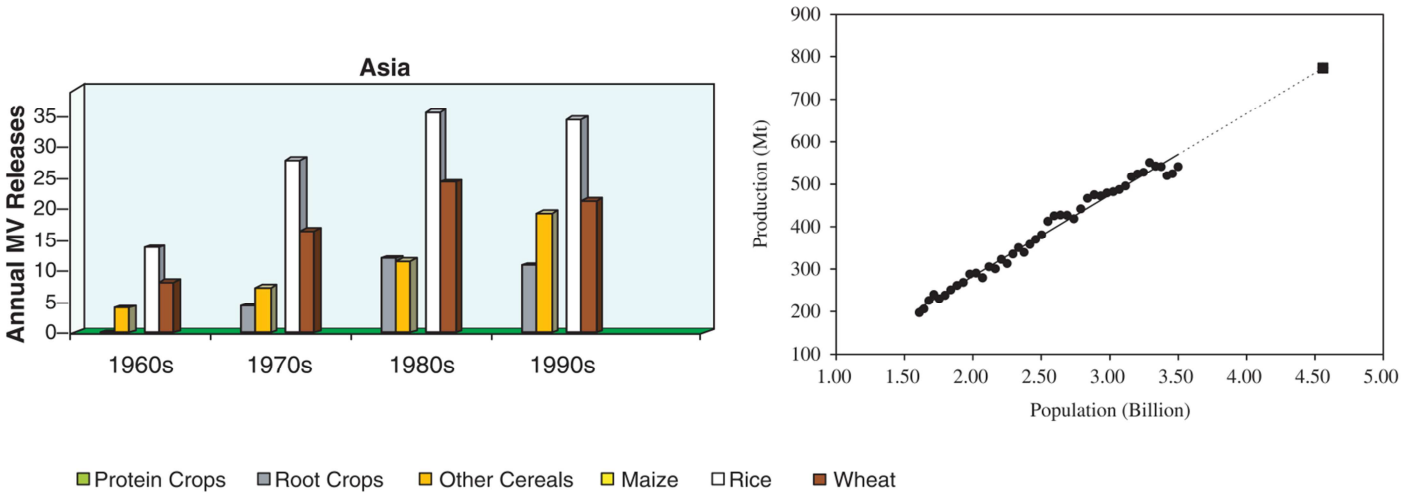

Fig. 1: Modern variety (MV) production in Asia by decade (adapted from (Evenson and Gollin, 2003) (left). Rice production and the population of Asia for the years 1961–2004. As an indication of the trend, the line is the regression of production on population, and the dotted line is an extrapolation to the population predicted for 2050 (square dot) (adapted from Mitchell and Sheehy (2006) (right).

Therefore, one approach of this study was to further elucidate the process of ROS formation and the occurrence of visible leaf symptoms under Zn deficiency (Chapter 2). Zn deficiency clearly induced oxidative stress in the plants, visualized by different staining methods already before the emergence of visible symptoms followed by an immediate antioxidant response. Throughout a series of different experiments under Zn deficient conditions with different plant genotypes we were able to show that oxidative stress is the main factor causing leaf damage and biomass reduction in rice under Zn deficiency (Chapter 1-3).

In 1993, a research project was started in Turkey (in co-operation with the NATO-Science for Stability Programme) to analyze high-yielding and Zn efficient cereal genotypes under Zn deficient conditions (Cakmak et al., 1999). This study confirmed that an efficient utilization

of Zn was rather a tolerance factor than enhanced Zn concentrations in the shoot. We were able to confirm that enhanced Zn translocation to the shoot is presumably not responsible for Zn effiency. The two genotypes tested under field and nutrient solution conditions did not differ in shoot Zn concentration (Frei et al., 2010; Chapter 1 and 2), as well as the mutant lines with increased sensitivity to Zn deficiency (Chapter 3). Nevertheless, different approaches such as genetic or agronomic biofortification are conceivable to increase Zn concentration in shoots and especially grains, since Zn deficiency in humans is a widespread problem especially in regions suffering from Zn deficient soils (Cakmak, 2008). However, a study with soils from Central Anatolia revealed that > 99 % of Zn added or present in soils is adsorbed and fixed by soil constituents (Cakmak, 1999), thus, the concentration of plant available Zn is extremely low. Application of Zn fertilizer is efficient only for a short time and associated with higher costs, which constitutes an obstacle especially for resource-poor farmers. Thus, the efficient internal utilization or enhanced ROS detoxification can be considered as an important tolerance factor against Zn deficiency.

1.2 Ascorbate and tolerance to zinc deficiency

Especially for plants it is important to control their redox status tightly, since photosynthetic processes favor the production of ROS and they are unable to change to a more harmless environment when exposed to certain stresses. Although it is well known that ROS detoxification can enhance plant performance under different stresses, exact knowledge is missing about which component of the ROS defense system can be targeted in the process of developing new rice cultivars tolerant to these stresses. This study revealed that an important part of the ROS defense system associated with tolerance to Zn deficiency and ozone stress is the ability to maintain a high AsA level. A correlation between AsA levels and Zn efficiency was given in a pair of genotypes, RIL46 and IR74 derived from a mapping population which exhibited contrasting tolerance in a field experiment (Frei et al., 2010). We were able to confirm this using the same genotypes in nutrient solution experiments as described in Chapter 1 and 2. However, several other factors possibly influenced tolerance to Zn deficiency. Since increased trehalose and proline concentrations were observed in the tolerant genotype, the question remained if AsA can function as a universal tolerance factor. Antioxidant enzyme activities did not differ between the two genotypes, however, there are several contradictory results from studies concerning the stress response to Zn deficiency. In

wheat cultivars, superoxide dismutase (SOD) activity decreased under Zn deficiency (Cakmak et al., 1997), whereas SOD activity was even higher in the two rice genotypes used in our study (Frei et al., 2010). The same contradicting results were found for ascorbate peroxidase (APX) activity, which was induced by Zn deficiency in rice (Frei et al., 2010) and wheat (Sharma et al., 2004), but reduced in beans (Cakmak and Marschner, 1993), demonstrating the complexity of the antioxidant response to Zn deficiency. Experiments with mutant lines with different AsA levels verified the important role of AsA in conferring tolerance to Zn deficiency (Chapter 2 and 3). All mutant lines tested in several experiments exhibited higher sensitivity to Zn deficiency related to lower shoot AsA levels, without differing in shoot Zn concentrations. As shown in Chapter 3, different AsA levels influenced enzyme activities taking part in the ascorbate-glutathione cycle, like APX and dehydroascorbate reductase (DHAR). Reduced AsA levels decreased especially APX activity in three out of four mutant lines, suggesting that its activity is dependent of the availability of AsA. Thus, it can be assumed that conflicting results regarding the stress response of different species under Zn deficiency, like induction or suppression of APX activity can be caused by different substrate availability of the plants.

A number of studies have confirmed the important role of AsA for different plant species and abiotic stresses apart from its involvement in conferring tolerance to Zn deficiency and ozone stress in rice. APX is induced in rice as a response to salt stress (NaCl) (Tsai et al., 2005) and a reduced AsA content was found to influence chilling tolerance of tomato fruits (Stevens et al., 2008). Drought-induced spikelet sterility of upland rice was lower in plants with higher AsA levels (Selote and Khanna-Chopra, 2004). Moreover, ozone tolerance was found to be associated with a higher AsA level in soybean (Michael Robinson and Britz, 2000) as well as in *Arabidopsis thaliana* (Conklin and Barth, 2004) and rice (Frei et al., 2012). Apart from its direct function as an antioxidant in the ascorbate-glutathione cycle, AsA is required for the reduction of other compounds involved in oxidative stress response, like violaxanthin de-epoxidase or α-tocopherol (Gill and Tuteja, 2010; Müller-Moulé et al., 2002) highlighting its diverse role in abiotic stress tolerance.

Many analyses and experiments concerning tolerance against Zn deficiency in rice are based on artificial conditions and are conducted with a limited number of genotypes or mutant lines. In Chapter 1, a pair of genotypes which was already tested under Zn deficient field conditions was used to elucidate possible tolerance factors, however, knowledge about the impact of AsA as a possible tolerance factor for plants with different genetic backgrounds was missing.

Thus, we conducted an experiment with 20 rice varieties showing contrasting tolerance against Zn deficiency derived from a field experiment at the International Rice Research Insitute (IRRI, Philippines) (unpublished results, Table 1). In this previous experiment, 251 varieties from the "aus" subgroup of rice and 9 core genotypes were grown on a Zn deficient field and phenotyped.

Table 1: Different rice varieties selected from a field experiment conducted at IRRI, Philippines on a zinc deficient soil. Field leaf bronzing score (LBS) and LBS in hydroponics under zinc deficient conditions were compared. The vigor score combines plant height and tiller number ranging from 1 (poor growth) to 5 (good growth).The ten best and ten worst performing lines are shown here and were selected for further experiments.

Line	Vigor score	field LBS	LBS in hydroponics
Kalubala vee	3,875	0,75	0,28
Jamir	4	1	0,89
Sreerampur shaita::irgc 64799-1	3,625	1	0,06
Dehula::irgc 74737-1	3,125	1	0,39
Karkati 87	3,5	1,25	0,17
Bawoi::irgc 34737-1	3,75	1,25	0,50
Gadra::irgc 73098-1	3,5	1,25	1,78
Fulkati::irgc 66783-1	2,375	1,375	0,50
Dular::irgc 636-1	3	1,375	0,44
Arc 6578	2,75	1,5	3,61
Arc 11755::irgc 21618-1	1,625	3	1,11
Gochi boro::irgc 66787-1	2,125	3	1,22
Rangpuri aus::irgc 66822-1	1,375	3	0,89
Santhi sufaid 207::irgc 28212-1	2,125	3	0,39
Uprh 184::irgc 61631-1	2,5	3	1,56
Maha pannithi::irgc 51021-1	2,25	3	1,11
9524	1,875	3,125	1,56
Safed macan::irgc 74775-1	1,75	3,125	5,17
29 a 2::irgc 28364-1	1,5	3,25	1,06
Habigonj boro 6::irgc 10873-1	2	3,25	1,78

The ten best and ten worst performing varieties from a Zn deficient field were selected for further analyses in a nutrient solution experiment under greenhouse conditions. The plants were subjected to control and Zn deficient conditions for three weeks and a leaf bronzing score (LBS) was assigned to the three youngest fully expanded leaves of each plant, respectively (as described in the materials and methods section in Chapter 2). Additionally,

plant height and weight were recorded, malondialdehyde (MDA) concentrations as an indicator for oxidative stress tolerance (data not shown) and AsA concentrations were measured. Visible leaf symptoms tend to be lower when plants are grown in nutrient solution compared to field-grown plants on low Zn soil (Frei et al., 2010; Wissuwa et al., 2006). Although Zn deficient soils contain more Zn than the -Zn nutrient solution used in our experiments (which contain no Zn at all), leaf bronzing is often stronger under field conditions. The reasons for this discrepancy are most likely other soil factors such as low redox potential, high pH and high bicarbonate in the soil solution (Ismail et al., 2007). The LBS under Zn deficiency in our results derived from a nutrient solution experiment was lower than the LBS from the same plants grown in low Zn soil. However, the average LBS from plants grown in hydroponics was higher in the field-intolerant genotypes than in the tolerant ones (Table 1). Thus, although the strength of symptoms differs between field and nutrient solution experiments, hydroponic culture can reflect a realistic stress response to Zn deficiency of different genotypes.

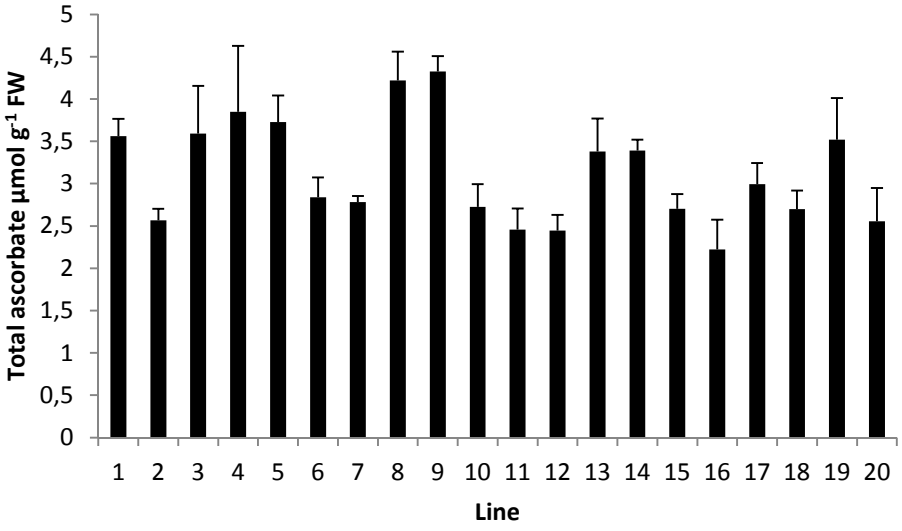

Fig. 2: Constitutive total ascorbate concentrations of the different varieties tested under field conditions regarding its tolerance to zinc deficiency in a previous experiment at the International Rice Research Institute (IRRI, Philippines). Ascorbate was measured from plants grown in nutrient solution for three weeks. Vertical bars indicate standard errors (n=6).

The AsA concentrations of the different genotypes showed great variations (Fig. 2), indicating that the 20-80 % lower AsA levels of our mutant lines described in Chapter 3 reflect natural variation of AsA in a good way. Experiments with AsA mutant lines described in Chapter 2 and 3 confirmed that plants with lower AsA levels are more sensitive to some abiotic stresses.

Our results demonstrated that AsA levels differ greatly amongst different varieties and that these differences can cause different oxidative stress responses in the field (Table 1, Fig. 2). The LBS derived from the field experiment correlated negatively with the constitutive AsA level of these plants (coefficient of determination R^2=0.21, p=0.04, significance of correlations was determined by a two-sample t-test). AsA is certainly not the only tolerance factor contributing to the performance of these varieties, however, it indicates that our results have the potential to enhance field performance and are not solely restricted to artificial greenhouse conditions.

1.4 The different roles of ascorbate

Apart from being involved in the antioxidant response to abiotic stresses, AsA levels influence different other plant functions, which possibly constitute one reason for its abundance in plants. AsA levels constantly increased during evolution of plants, which Gest et al. (2013) explained with a cost-benefit analysis. For example, an *Arabidopsis thaliana* mutant with lower AsA level entered senescence earlier than its wild-type, indicating an additional benefit for plants with higher AsA levels (Conklin and Barth, 2004). Our studies confirmed the impact of AsA on plant fitness, like biomass production, photosynthetic capacity, germination rates and especially yield parameters.

Maintaining a high photosynthetic rate is of great importance for plants and can provide a good basis for high-yielding varieties (Peng et al., 2008). Evidence exists that improved photosynthesis especially during the late reproductive phase is closely associated with higher yield potential in rice (Takai et al., 2006). Models predict that the increases in rice yield required to feed a growing world population can only be achieved by enhanced efficiency of photosynthesis (Long et al., 2006; Mitchell and Sheehy, 2006). In this context, several researchers proposed to incorporate the C_4 pathway into rice, which might lead to a potential increase of photosynthesis efficiency up to 50 % (Hibberd et al., 2008; Mitchell and Sheehy, 2006). The mutant lines analyzed in Chapter 3 with lower AsA levels in our study had a reduced photosynthetic capacity, accompanied by decreased yield parameters. However, further research is needed to determine if the photosynthesis rate and yield parameters are directly dependent on AsA or just an effect of a poor plant performance in general due to lower AsA levels.

Seed maturation, grain filling and germination was influenced by different AsA levels as described in Chapter 3 and Table 2. Biomass production was impaired in plants with lower AsA levels under Zn deficiency, as well as rough rice production and the proportion of filled grain in mutant lines even under non-stressed conditions. One the one hand, AsA can directly influence the process of seed maturation and yield parameters; on the other hand, due to its protective role against oxidative stress, it can avoid yield loss resulting from abiotic stresses. As mentioned before, Zn deficiency can severely affect yields of important crops. Even without showing visible symptoms of Zn deficiency, the quality of crop products and yields can be reduced (so-called "hidden deficiency") (Alloway, 2009). In this context, the antioxidant function of AsA is most likely the most important one during grain filling and seed maturation. A proteome analyses in barley revealed that the grain filling phase is characterized by an accumulation of enzymes taking part in the ROS defense system such as APX (Finnie et al., 2002). Seeds are particularly exposed to different stresses, for example to drought stress during development and maturation (Kranner et al., 2010). Therefore, they must possess high tolerance against stress.

As described in Chapter 3, a lower AsA shoot level resulted in lower AsA levels in the panicles and in dried seeds (Fig. 3). This correlated with lower germination rates and higher spikelet sterility, confirming the important role of AsA for grain production. Especially the germination rate of a mutant line with a knock-out in the gene encoding for GDP-L-galactose phosphorylase (GGP, also known as VTC2) with an extremely low AsA level was affected (Table 2, Chapter 3). Transplanting is the most popular method for rice cultivation, in which seeds are in some cases presoaked, germinated in a nursery and then transplanted to a paddy field. However, these methods can result in poor germination, growth and limited yields, thus, different methods to improve germination rates and time are required (Basra et al., 2005; Khan, 1992; Lee et al., 1998). In this context, seed priming with AsA was found to improve growth and yield of rice plants (Farooq et al., 2007). The majority of AsA in dried seeds was present in its oxidized form (Fig. 3), confirming the results of De Gara et al. (1997) who found that dry grains of wheat contain no reduced AsA at all and only a small amount of oxidized AsA. In the case of wheat, AsA biosynthesis starts after 8-20 hour of germination, however, AsA is required even before and sufficient AsA must be provided by reduction of the stored deydroascorbate (DHA) (De Gara et al., 1997). Thus, the impact of a higher AsA level on seedling development and a possible replacement of pre-soaking methods by enhanced seedling performance can be an interesting topic for further research.

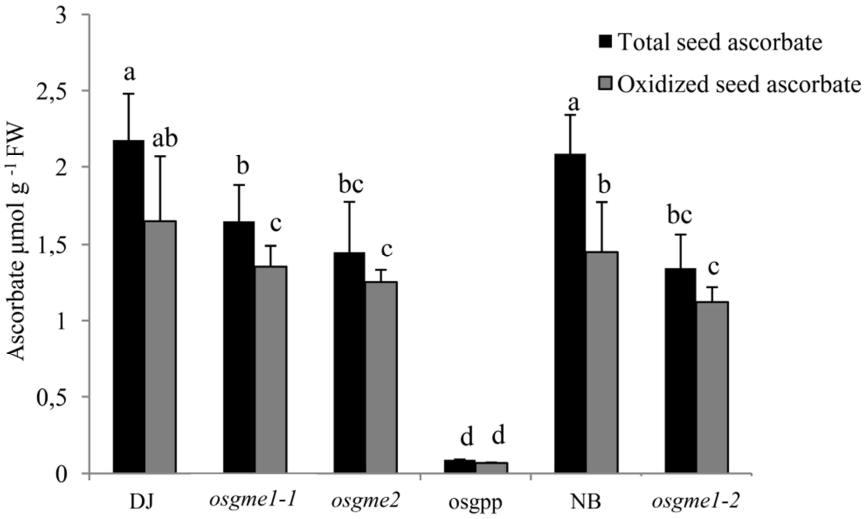

Fig. 3: Total (black bars) and reduced (grey bars) ascorbate in dried seeds of the mutant lines and their wildtypes. Seeds were taken from plants grown in soil described in Chapter 3 and dried at 50°C for three days after harvesting. For the ascorbate measurements, several seeds from one plant were dehulled and pooled to reach a minimum of 60 mg per sample. Data bars not sharing the same superscript letter are statistically different at $P < 0.05$ by LSD-test. Vertical bars indicate standard errors (n=4).

Table 2: Germination rates of the mutant lines and their corresponding wild-types. In total, 50 seeds per genotype were germinated

	Germination rate (%)
DJ	89
osgme1-1	73
osgme2	71
osggp	33
NB	87
osgme1-2	65

1.3 Ascorbate biosynthesis

Additionally to its function as an antioxidant, the AsA metabolism was investigated in detail in this study. The results presented in Chapter 1 revealed that AsA biosynthesis is presumably the limiting factor in maintaining a sufficient AsA level under Zn deficiency. This was confirmed by different mutant lines with suppressed biosynthetic genes resulting in reductions

of the AsA pool (Chapter 2 and 3). Expression analyses of all of the annotated biosynthetic genes of the predominant D-mannose/L-galactose pathway in the contrasting genotypes RIL46 and IR74 suggested that presumably not a single gene is responsible for maintaining a higher biosynthetic rate of AsA (Chapter 1). However, a knock-out of the two genes *GDP-mannose-3,5-epimerase* (*GME*) and *GGP*, considered to play an important regulating role for AsA biosynthesis, had very differential effects on the AsA level of the mutant lines, as seen in Chapter 2 and 3. A knock-out of *OsGME* had a moderate effect on the AsA level, however it had a significant impact on stress tolerance and especially on the yield of these mutants. GME is considered as the most highly conserved protein in AsA biosynthesis among monocotyledonous and dicotyledonous plants (about 90 % identity) (Wolucka and Van Montagu, 2007). Mapping of candidate genes in AsA metabolism in a quantitative trait loci (QTL) mapping approach in tomato revealed a colocalization of a gene encoding for GME and a QTL identified as a region controlling AsA content (Stevens et al., 2007). Moreover, GME is generally considered as an important enzyme in AsA biosynthesis, since it forms the branch of the predominant pathway with an alternative pathway via L-gulose and acts as a control point for carbon flux into AsA and cell wall biosynthesis (Valpuesta and Botella, 2004; Wolucka and Van Montagu, 2003). RNAi-silencing of GME in tomato confirmed the impact on cell wall composition, since plants suffered from severe growth defects which could not be restored by AsA supplementation (Gilbert et al., 2009). Analyses of cell wall components revealed significant changes in sugars such as mannose and galactose. However, the impact of differentially expressed genes encoding for GME in rice on cell wall composition and the resulting changes in growth of the plants remains to be elucidated.

Our studies emphasized the important role of *OsGGP* in AsA biosynthesis, since a knock-out resulted in extremely reduced AsA levels and plants were hardly able to survive (Chapter 3). Developmental fluctuations of the AsA pool were missing in these mutants, especially when wild-type plants increased AsA levels shortly before entering the reproductive stage. The onset of flowering was strongly affected by AsA supporting the hypothesis of a regulatory role of *OsGGP* during the reproductive stage. This confirmed the results of a study by Kotchoni et al. (2009), who were able to establish a significant association between AsA levels and flowering time in *Arabidopsis thaliana*. There are several reasons to consider *GGP* as an important regulator of AsA levels. It is the first step solely committed to AsA biosynthesis (Smirnoff and Wheeler, 2000), and several studies confirmed its strong induction by high light (Müller-Moulé, 2008; Yabuta et al., 2007). GGP was the last identified enzyme

in the D-mannose/L-galactose pathway (Laing et al., 2007), and thus, detailed knowledge about its contribution and regulation of AsA biosynthesis in different species is missing. However, different studies reported severe alterations in AsA content by manipulation of *GGP*. Transient expression of a kiwifruit homologue of *GGP* in tobacco leaves resulted in a more than 3-fold increase of AsA concentration in leaves (Laing et al., 2007) and double knock-out mutants of the two homologues of *GGP* in *Arabidopsis thaliana* were unable to grow without exogenous AsA supply (Dowdle et al., 2007).

Another interesting aspect is that the final enzyme of AsA biosynthesis, L-galactono-1,4-lactone dehydrogenase, is located at the inner mitochondrial membrane and that cytochrom c is the electron acceptor in this enzymatic reaction (Bartoli et al., 2000). Expression of the two isoforms of the ecoding gene was enhanced in RIL46 compared to the intolerant IR74 to a greater extend than most of the other genes of this pathway (Chapter 1). Evidence exists that AsA biosynthesis is dependent on the electron flow through mitochondrial complex I and respiration in *Arabidopsis thaliana* (Millar et al., 2003). We detected significant changes in intermediates of the tricarboxylic acid (TCA) cycle like succinate, malate and isocitrate in the AsA deficient mutant lines (Chapter 3, Supplemental data), however, the relation between the AsA level and the TCA cycle warrants further investigations. Since stress tolerance and the cells redox homeostasis is associated with mitochondrial function (Dutilleul et al., 2003), the link between AsA biosynthesis, mitochondrial function and stress tolerance might be an interesting topic for further research.

1.4 Outlook

This study answered the questions how plants are able to increase AsA levels and if AsA can protect rice plants from different stresses and increase plant fitness and yield. However, some results raised further questions which will be interesting to investigate.

Ascorbate transport from shoots to roots

In Chapter 2, we demonstrated that AsA is present in much lower concentrations in roots compared to shoots. If AsA is synthesized predominantly in photosynthetic tissue, it would result in less capacity to transport AsA from source to sink tissue in plants with lower AsA levels. However, the transport of AsA is still not understood in detail. Some investigations have been made regarding the subcellular transport of AsA. Since the last step of AsA

biosynthesis occurs at the inner mitochondrial membrane, AsA is presumably transported to the cytosol via simple diffusion (Horemans et al., 2000). In the chloroplast, AsA is needed in high concentrations and there is evidence that this transport is carrier-mediated (Anderson et al., 1983; Beck et al., 1983). Only recently an AsA transporter at the chloroplast envelope membrane was found in *Arabidopsis thaliana* (Miyaji et al., 2015). AsA movement across the plant cell membrane is mediated by membrane-associated carriers with high affinities for AsA and DHA (Horemans et al., 1998; Rautenkranz et al., 1994). This transport is essential for plants, since no extracellular AsA biosynthesis exists and the apoplast contains around 10 % of the AsA pool. However, the movement of AsA from source (shoot) to sink (root) tissue by phloem mass flow is not well investigated. Franceschi and Tarlyn (2002) showed that long-distance transport of AsA is working, however, it is not known, if sufficient AsA supply in roots under stress situations is mediated by enhanced local biosynthesis or long-distance transport from shoot tissue. It has been confirmed that AsA biosynthesis is working in roots for *Cucurbita maxima* (Groten et al., 2005; Liso et al., 2004; Matamoros et al., 2006), but information whether this is also true for rice is lacking. Thus, research is needed to further investigate the long-distance transport of AsA and its impact on root tolerance against oxidative stress, as well as the capacity of rice roots to synthesize AsA independently.

The role of GGP

A knock-out of the *OsGGP* gene had a severe effect on AsA level (80-90 % reduction), although only one homologue out of two was affected. As mentioned in Chapter 3, we hypothesized that OsGGP might play a regulating role for the onset of flowering. In a gene expression study of *GGP* in *Arabidopsis thaliana*, a constant increase of *GGP* expression in wild-type plants throughout the plant development was observed (Müller-Moulé, 2008), however, developmental changes of AsA levels in *Arabidopsis thaliana* differ from that in rice. Thus, gene expression analyses of *GGP* in heterozygous rice *osggp* mutants and their wild-types used in Chapter 3 might give further information. Another interesting fact is that a GGP:YFP fusion protein used in the study of Müller-Moulé (2008) in *Arabidopsis thaliana* was not only found in the cytosol but also in the nucleus. AsA biosynthesis occurs in the cytosol, except for the last step, which is located in the inner mitochondrial membrane, thus, the location of GGP in the cytosol is expected. Since the fusion product of GGP with YFP is too big to simply diffuse into the nucleus, GGP is likely to possess a nuclear localization signal. Exact explanations about the reasons for the presence of GGP in the nucleus are

lacking, however, it is conceivable that GGP act as a dual-function protein with an additional regulatory role. Apart from their enzymatic function, these proteins can regulate gene expression by entering the nucleus e.g. under changing environmental conditions. In the case of GGP, it is possible that high light conditions trigger a change of localization. However, to confirm these hypotheses further research is necessary.

Other tolerance factors

Apart from AsA, other factors can confer tolerance to Zn deficiency, possibly in combination with enhanced AsA levels. In this study, a higher proline and trehalose content was found in the tolerant genotype RIL46 (Chapter 1). Whether trehalose is efficient in protecting plants from oxidative stress is not clear yet (Wingler and Wingler, 2002), thus, a higher proline content might be the more promising factor to increase oxidative stress tolerance. Proline is widely known as an osmolyte protecting plants from salt or drought stress, but apart from that, it might have positive effects on membrane and enzyme activity (Ashraf and Foolad, 2007). If the increase of proline is a cause or effect of Zn deficiency deserves further attention.
Concerning shoot-mediated tolerance, different other mechanisms have been proposed in the literature. It was found that Zn efficient wheat genotypes often differ in their ability to maintain activities of Zn-dependent enzymes (Hacisalihoglu et al., 2003). Carbonic anhydrase (CA) is one Zn dependent enzyme showing decreased activity under Zn deficiency in rice and wheat (Rengel and Graham, 1995; Sasaki et al., 1998) and it was suggested that tolerant genotypes with higher CA activity are capable of maintaining higher photosynthesis rates under Zn deficiency by mediating CO_2 movement and fixation. Another important enzyme requiring Zn is Cu/Zn SOD, involved in the ROS detoxification system. In different studies with wheat, rye and black gram (*Vigna mungo*), a positive correlation of Cu/Zn SOD and Zn efficiency was confirmed (Cakmak and Marschner, 1993; Cakmak et al., 1997; Hacisalihoglu et al., 2003; Pandey et al., 2002). This strengthens the suggestion that several tolerance factors might work together in different crop cultivars tolerant to Zn deficiency.

In conclusion AsA biosynthesis represents a promising target for the improvement of rice performance under abiotic stress conditions. The influence of AsA on photosynthesis, biomass production and yields even under non-stressed conditions might be an additional benefit.

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Supplemental Data

Chapter 1

Table S1: Gene specific primers used for quantitative RT-PCR

Gene	**Locus**	Primer forward	Primer reverse
Mannose-6-phosphate isomerase	Os01g0127900	TCGCTGGAGAAACAATACCC	CAAACCAGCACGAACAACAT
GDP Mannose pyrophosphorylase	Os01g0847200	CAAGATCAATGCGGGAATTT	CCAACATCCATCCAAAAACC
GDP Mannose pyrophosphorylase	Os08g0237200	CGAATCTCAGCTGATGCAAA	TGTGTGCTCCAGTGGCTAAC
GDP Mannose pyrophosphorylase	Os03g0268400	CAAGGACTTCGAGAGCAAGC	GGGACTTGTGGAACTGGATG
GDP-mannose 3,5-epimerase	Os10g0417600	ATTCTGCAGGAAGGCTCAGA	TTCATCGCTTCCAATGTTCA
GDP-mannose 3,5-epimerase	Os11g0591100	GATGCCTATGGCTTGGAAAA	CAAAACGGTCAGTGGAGGTT
VTC2 (GDP-L-galactose phosphorylase)	Os12g0190000	AGTGCCTTTCCCTGTTGAGA	ATACAAGCGCTGGAAACCAC
GDP-L-galactose phosphorylase	Os01g0901300	TGTTGCAAGTGAAGCAAAGG	ACATCGGACAAATCCTCCAG
L-galactose-1P- phosphatase	Os03g0587000	TACCCGGACCACAAGTTCAT	TCCACGAACAGCTGTGAAAA
L-galactose dehydrogenase	Os12g0482700	GCTGCCATTGAGCATCTACA	TTGCAAGAGGTGAAGCACTG
L-galactono-1,4-lactone dehydrogenase	Os11g0143500	ATCTTGCTCGCTGTGGACTT	GGAGGATTGCATTGGACAAC
L-galactono-1,4-lactone dehydrogenase	Os12g0139600	ATGCTCGACAAAGGAAGGAA	GTTAGGGTCAAGCTCCATGC
Myo-inositol oxygenase	Os06g0561000	ACCCAAAGCTCAACACCAAGT	CCTTGTCCTCATCGTTCATCAGATGC
U2 snRNP auxiliary factor, small subunit	Os05g0564200	CACAACAGGCCAACTGTGTC	GAGGGTCTCAACCTCACCAAACTT

Table S2: Complete set of metabolomic, gene expression, and enzyme activity data of two contrasting rice genotypes exposed to + and - Zn treatments for 24 days

	IR74 + Zn			RIL46 + Zn		
	Day 2	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**
Zinc (µg g-1)	131,2	121,6	109,7	119,8	87,0	117,8
Leaf bronzing score	0,0	0,0	0,0	0,0	0,0	0,0
Malondialdehyde (nmol ml-1 g-1 FW)	11,6	13,0	17,7	10,9	10,9	13,5
Total Ascorbate (µmol g-1 FW)	6,5	6,9	7,0	7,0	6,7	7,0
Reduced Ascorbate (µmol g-1 FW)	5,9	6,7	6,8	6,6	6,2	6,9
Total Glutathione (µmol g-1 FW)	0,2	0,3	0,3	0,2	0,2	0,2
Reduced Glutathione (µmol g-1 FW)	0,1	0,1	0,1	0,1	0,1	0,1
DHAR activity (µmol ml-1 g-1 FW)	0,184	0,202	0,1825	0,212	0,182	0,1875
PMI activity (µmol min-1 mg-1 FW)		4,0275	2,8975		4,7725	3,1725
Amino acids						
Aspartic acid (nmol g-1 FW)	1242,1	1223,7	1502,7	1103,7	1269,2	1070,2
Glutamic acid (nmol g-1 FW)	2444,9	2301,5	2994,0	2353,2	2437,5	2430,1
Serine (nmol g-1 FW)	1183,6	1081,1	1840,6	1076,9	1478,7	1297,2
Asparagine (nmol g-1 FW)	7937,1	1270,5	1519,3	6070,8	1482,7	1811,0
Glycine (nmol g-1 FW)	407,5	361,7	334,7	402,4	274,2	291,6
Glutamine (nmol g-1 FW)	3201,8	869,3	1090,6	2397,2	730,6	903,6
Histidine (nmol g-1 FW)	51,8	30,5	33,8	48,6	33,8	32,7
Threonine (nmol g-1 FW)	558,4	340,3	451,7	546,8	383,9	385,1
Arginine (nmol g-1 FW)	282,6	91,9	79,2	232,8	56,8	72,3
Alanine (nmol g-1 FW)	953,1	679,6	897,0	954,3	737,7	765,7
Proline (nmol g-1 FW)	71,1	74,0	85,8	89,4	100,5	102,0
Tyrosine (nmol g-1 FW)	41,4	53,7	51,6	43,8	49,4	48,7
Valine (nmol g-1 FW)	200,1	129,1	142,0	151,5	115,3	116,0
Methionine (nmol g-1 FW)	10,4	28,3	21,8	22,4	16,8	30,7
Isoleucine (nmol g-1 FW)	40,0	26,0	29,0	39,0	34,7	29,9
Lysine (nmol g-1 FW)	94,1	49,8	58,6	76,3	59,9	40,4
Leucine (nmol g-1 FW)	53,7	36,3	48,1	45,3	45,5	39,0
Phenylalanine (nmol g-1 FW)	34,2	41,9	42,8	31,3	38,4	37,0
Metabolites						
Threonate (nmol/g)	39,2	52,0	58,6	49,5	52,7	50,4
Mannose 1-P (nmol/g)	10,5	11,6	9,6	10,2	11,9	10,0
GDP-Mannose(nmol/g)	2,0	1,5	1,3	1,4	1,2	1,3
Glucuronic acid (nmol/g)	4,8	7,0	5,7	5,7	8,1	5,8
Trehalose-6-P (nmol/g)	1,6	1,5	1,5	1,6	1,4	1,2
Sucrose-6-P (nmol/g)	1,6	2,1	2,1	1,8	1,8	2,0
Succinate (nmol/g)	0,9	0,7	0,9	0,9	0,8	1,1

	IR74 + Zn			RIL46 + Zn		
	Day 2	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**
Oxoglutarate (nmol/g)	0,3	0,2	0,3	0,3	0,1	0,2
Glucose-6-P (nmol/g)	3,4	3,8	3,6	3,8	3,2	3,3
Fructose-6-P (nmol/g)	3,4	3,0	2,9	2,3	0,4	0,9
Oxalate (nmol/g)	73,8	59,5	148,3	108,7	34,0	66,2
Ribose-5-P (nmol/g)	0,1	0,2	0,4	1,4	0,2	0,9
ADP-Glucose (nmol/g)	0,4	0,5	0,5	0,4	0,5	0,2
3PGA (nmol/g)	17,1	27,4	33,6	23,2	13,4	21,2
PEP (nmol/g)	34,9	53,2	61,0	50,0	47,8	44,8
UDP-Glucose (nmol/g)	42,0	33,1	47,1	30,2	23,2	26,1
Trans-Aconitate (nmol/g)	11,1	3,4	3,9	11,2	5,6	4,6
Cis-Aconitate	0,7	0,6	0,7	0,3	0,5	0,5
Fru1,6bisP	0,5	1,5	0,5	0,4	0,6	0,5
UDP	9,6	2,7	3,8	3,0	5,1	3,5
Malate (µmol/g)	1,7	2,3	1,8	1,5	1,9	1,8
Citrate (µmol/g)	1,7	1,5	1,3	1,2	1,3	1,5
Isocitrate (nmol/g)	74,5	85,4	117,2	84,8	110,2	123,0
Glucose (nmol/g)	577,5	810,0	813,8	1157,5	1414,0	2042,0
Fructose (nmol/g)	822,5	802,0	1022,5	1275,0	1457,5	2302,0
Saccharose (nmol/g)	8379,0	14390,0	17145,0	10583,0	14186,0	16331,0
Inositol (nmol/g)	443,5	578,4	803,0	624,3	505,7	635,7
Glycerol (nmol/g)	49,2	105,8	19,1	239,4	105,4	45,3
Erythritol (nmol/g)	203,0	259,1	313,4	222,2	247,3	258,6
Trehalose (nmol/g)	42,0	87,3	69,7	50,5	79,6	76,9
Galactose (nmol/g)	251,3	250,2	261,2	250,9	177,2	211,8
Rhamnose (nmol/g)	34,9	29,9	20,9	29,4	25,6	22,6
Gene expression data						
Mannose-6-phosphate isomerase	1,0	0,5	0,7	0,6	1,2	0,6
GDP Mannose pyrophosphorylase	1,0	0,8	1,4	1,0	0,6	0,9
GDP Mannose pyrophosphorylase	1,0	1,1	1,2	0,4	1,2	1,1
GDP Mannose pyrophosphorylase	1,0	0,3	0,4	0,5	1,1	1,3
GDP-mannose 3,5-epimerase	1,0	0,6	0,9	0,4	1,0	1,1
GDP-mannose 3,5-epimerase	1,0	1,2	1,0	0,7	3,7	1,8
VTC2 (GDP-L-galactose phosphorylase)	1,0	1,2	2,4	1,2	0,9	1,9
GDP-L-galactose phosphorylase	1,0	1,1	0,9	0,6	1,3	1,3
L-galactose-1P- phosphatase	1,0	1,0	2,2	1,7	1,2	1,8
L-galactose dehydrogenase	1,0	0,8	0,9	0,6	1,3	1,2
L-galactono-1,4-lactone dehydrogenase	1,0	1,3	2,0	1,3	0,8	1,3
L-galactono-1,4-lactone dehydrogenase	1,1	0,9	0,8	0,5	1,6	1,8
***Myo*-inositol oxygenase**	1,0	1,2	0,8	0,3	0,6	1,0

	IR74 - Zn			RIL46 - Zn		
	Day 2	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**
Zinc (µg g-1)	26,3	25,3	15,3	21,9	7,6	13,1
Leaf bronzing score	0,0	1,5	1,4	0,0	0,0	0,0
Malondialdehyde (nmol ml-1 g-1 FW)	12,4	19,4	25,3	12,2	16,1	13,8
Total Ascorbate (µmol g-1 FW)	7,2	7,1	6,2	7,2	7,6	8,1
Reduced Ascorbate (µmol g-1 FW)	6,7	6,9	5,6	6,7	7,2	7,5
Total Glutathione (µmol g-1 FW)	0,2	0,5	0,2	0,2	0,3	0,2
Reduced Glutathione (µmol g-1 FW)	0,0	0,2	0,1	0,0	0,1	0,1
DHAR activity (µmol ml-1 g-1 FW)	0,2225	0,2025	0,1825	0,216	0,1875	0,1725
PMI activity (µmol min-1 mg-1 FW)		3,1525	2,9		3,9325	2,7975
Amino acids						
Aspartic acid (nmol g-1 FW)	1591,3	1121,1	1509,9	1339,0	1256,6	1429,7
Glutamic acid (nmol g-1 FW)	2845,0	1842,9	2931,0	2569,7	2252,7	2323,5
Serine (nmol g-1 FW)	1188,8	8239,2	3165,6	1046,1	4602,6	2802,4
Asparagine (nmol g-1 FW)	3333,4	8718,3	10883,3	4199,8	5834,2	5542,1
Glycine (nmol g-1 FW)	359,1	1359,3	808,6	400,1	667,8	685,8
Glutamine (nmol g-1 FW)	1983,1	12749,7	7127,7	2566,3	7652,7	5043,2
Histidine (nmol g-1 FW)	46,0	139,6	215,6	43,9	128,9	149,4
Threonine (nmol g-1 FW)	545,9	1405,3	1073,7	497,5	1273,6	1052,8
Arginine (nmol g-1 FW)	136,6	1135,0	781,6	96,6	510,4	417,2
Alanine (nmol g-1 FW)	981,8	3347,5	1476,9	965,5	3270,2	2167,9
Proline (nmol g-1 FW)	79,0	229,7	174,7	97,0	347,4	805,2
Tyrosine (nmol g-1 FW)	51,0	44,2	61,4	46,4	50,6	62,4
Valine (nmol g-1 FW)	219,7	613,1	387,1	167,8	432,7	349,8
Methionine (nmol g-1 FW)	30,0	75,8	23,5	11,5	49,1	32,8
Isoleucine (nmol g-1 FW)	47,3	129,8	128,8	43,5	119,0	103,6
Lysine (nmol g-1 FW)	73,8	192,3	330,2	53,4	229,1	195,3
Leucine (nmol g-1 FW)	53,8	134,8	170,0	45,7	126,9	102,2
Phenylalanine (nmol g-1 FW)	38,0	66,0	137,2	33,0	65,9	122,4
Metabolites						
Threonate (nmol/g)	53,8	14,6	20,7	46,8	12,8	15,5
Mannose 1-P (nmol/g)	10,4	10,1	8,0	10,4	7,7	7,7
GDP-Mannose(nmol/g)	2,1	1,5	1,1	1,7	0,9	0,7
Glucuronic acid (nmol/g)	6,6	4,6	3,9	8,1	5,9	4,2
Trehalose-6-P (nmol/g)	1,8	1,1	0,5	1,4	0,9	1,1
Sucrose-6-P (nmol/g)	1,6	2,5	1,8	1,9	2,6	2,0
Succinate (nmol/g)	1,0	0,6	0,9	1,0	0,6	0,5
Oxoglutarate (nmol/g)	0,4	0,2	0,3	0,2	0,1	0,1
Glucose-6-P (nmol/g)	3,5	2,4	2,5	3,6	1,9	2,1
Fructose-6-P (nmol/g)	3,9	1,2	3,5	2,0	0,0	1,4
Oxalate (nmol/g)	111,1	22,8	52,8	101,1	31,5	40,2
Ribose-5-P (nmol/g)	1,7	0,2	1,0	1,4	0,2	0,4

	IR74 - Zn			RIL46 - Zn		
	Day 2	**Day 16**	**Day 24**	**Day 2**	**Day 16**	**Day 24**
ADP-Glucose (nmol/g)	0,4	0,2	0,7	0,6	0,2	0,6
3PGA (nmol/g)	21,3	9,5	11,0	20,0	4,4	6,3
PEP (nmol/g)	45,4	26,0	23,7	44,6	27,1	20,4
UDP-Glucose (nmol/g)	38,5	31,8	32,4	30,9	24,4	24,0
Trans-Aconitate (nmol/g)	9,7	11,0	8,9	6,3	16,7	9,7
Cis-Aconitate	0,6	0,7	0,7	0,4	0,4	0,4
Fru1,6bisP	1,3	0,3	0,3	0,6	0,2	0,2
UDP	2,6	2,6	3,4	2,9	2,4	3,4
Malate (µmol/g)	1,7	1,6	1,7	2,1	1,8	2,1
Citrate (µmol/g)	1,5	3,7	4,1	2,1	4,0	5,0
Isocitrate (nmol/g)	110,6	185,8	309,5	253,5	277,0	316,9
Glucose (nmol/g)	433,0	475,0	912,0	932,5	835,0	1150,0
Fructose (nmol/g)	612,5	728,0	940,0	1150,0	1000,0	852,5
Saccharose (nmol/g)	7556,3	19560,0	12461,3	9853,8	19336,3	15575,0
Inositol (nmol/g)	398,0	545,0	448,5	617,6	709,1	647,2
Glycerol (nmol/g)	188,6	137,3	28,6	139,8	261,3	66,0
Erythritol (nmol/g)	161,9	396,7	444,0	285,6	464,3	738,6
Trehalose (nmol/g)	49,8	40,8	24,8	86,5	87,2	68,7
Galactose (nmol/g)	239,0	1870,1	766,4	249,6	898,0	643,1
Rhamnose (nmol/g)	22,3	29,1	43,0	27,0	45,1	60,0
Gene expression data						
Mannose-6-phosphate isomerase	1,1	0,5	0,8	0,8	1,4	1,7
GDP Mannose pyrophosphorylase	1,6	1,4	1,6	1,7	1,5	1,9
GDP Mannose pyrophosphorylase	0,9	0,4	0,6	2,0	0,6	0,5
GDP Mannose pyrophosphorylase	0,8	0,8	1,1	3,3	1,2	1,5
GDP-mannose 3,5-epimerase	1,0	0,7	0,8	0,5	0,9	1,5
GDP-mannose 3,5-epimerase	1,2	1,1	1,5	1,6	3,0	2,0
VTC2 (GDP-L-galactose phosphorylase)	1,9	1,3	2,2	1,8	1,4	2,2
GDP-L-galactose phosphorylase	1,1	0,9	1,3	1,4	1,0	1,7
L-galactose-1P- phosphatase	0,7	0,8	2,1	2,0	0,6	0,9
L-galactose dehydrogenase	1,3	2,0	1,5	2,6	2,0	2,5
L-galactono-1,4-lactone dehydrogenase	1,6	2,2	2,0	1,4	3,3	2,0
L-galactono-1,4-lactone dehydrogenase	1,3	2,0	1,1	1,0	2,5	1,3
***Myo*-inositol oxygenase**	1,0	0,8	2,1	3,2	4,2	2,9

	ANOVA p-vaules					
	Treatment (T)	**Genotype (G)**	**Day (D)**	**TxG**	**TxD**	**TxGxD**
Zinc (µg g-1)	0,000	0,012	0,026	n.s	n.s	n.s
Leaf bronzing score	0,000	0,000	0,000	0,000	0,000	0,000
Malondialdehyde (nmol ml-1 g-1 FW)	0,000	0,000	0,000	n.s	0,045	n.s
Total Ascorbate (µmol g-1 FW)	n.s	0,022	n.s	0,041	n.s	n.s
Reduced Ascorbate (µmol g-1 FW)	n.s	n.s	n.s	n.s	n.s	n.s
Total Glutathione (µmol g-1 FW)	0,000	0,000		n.s		0,000
Reduced Glutathione (µmol g-1 FW)	n.s	0,000		n.s		0,000
DHAR activity (µmol ml-1 g-1 FW)	n.s	n.s	0,280	n.s	n.s	n.s
PMI activity (µmol min-1 mg-1 FW)	0,025	0,063	0,000	n.s	n.s	n.s
Amino acids						
Aspartic acid (nmol g-1 FW)	0,006	0,016	0,032	n.s	0,036	n.s
Glutamic acid (nmol g-1 FW)	n.s	n.s	0,004	n.s	n.s	n.s
Serine (nmol g-1 FW)	0,000	0,000	0,000	0,000	0,000	0,000
Asparagine (nmol g-1 FW)	0,000	0,000	0,038	0,004	0,000	0,000
Glycine (nmol g-1 FW)	0,000	0,000	0,000	0,000	0,000	0,000
Glutamine (nmol g-1 FW)	0,000	0,000	0,000	0,000	0,000	0,000
Histidine (nmol g-1 FW)	0,000	0,001	0,000	0,001	0,000	0,001
Threonine (nmol g-1 FW)	0,000	n.s	0,000	n.s	0,000	n.s
Arginine (nmol g-1 FW)	0,000	0,000	0,000	0,001	0,000	0,023
Alanine (nmol g-1 FW)	0,000	n.s	0,000	n.s	0,000	0,007
Proline (nmol g-1 FW)	0,000	0,000	0,000	0,000	0,000	0,000
Tyrosine (nmol g-1 FW)	0,026	n.s	0,000	n.s	0,006	n.s
Valine (nmol g-1 FW)	0,000	0,000	0,000	0,019	0,000	0,015
Methionine (nmol g-1 FW)	0,000	n.s	0,000	0,007	0,000	n.s
Isoleucine (nmol g-1 FW)	0,000	n.s	0,000	0,026	0,000	n.s
Lysine (nmol g-1 FW)	0,000	0,000	0,000	0,018	0,000	0,000
Leucine (nmol g-1 FW)	0,000	0,000	0,000	0,000	0,000	0,001
Phenylalanine (nmol g-1 FW)	0,000	0,022	0,000	n.s	0,000	n.s
Metabolites						
Threonate (nmol/g)	0,000	n.s	0,043	n.s	0,000	n.s
Mannose 1-P (nmol/g)	0,000	n.s	0,002	n.s	0,010	n.s
GDP-Mannose(nmol/g)	n.s	0,000	0,000	n.s	n.s	n.s
Glucuronic acid (nmol/g)	n.s	n.s	0,037	n.s	0,002	n.s
Trehalose-6-P (nmol/g)	0,000	n.s	0,000	n.s	0,008	0,004
Sucrose-6-P (nmol/g)	0,031	n.s	0,000	n.s	0,001	n.s
Succinate (nmol/g)	n.s	n.s	0,004	n.s	n.s	n.s
Oxoglutarate (nmol/g)	n.s	0,000	0,000	n.s	n.s	n.s
Glucose-6-P (nmol/g)	0,000	n.s	0,000	n.s	0,001	n.s
Fructose-6-P (nmol/g)	n.s	0,000	0,000	n.s	n.s	n.s
Oxalate (nmol/g)	n.s	n.s	0,003	n.s	0,036	n.s
Ribose-5-P (nmol/g)	0,009	n.s	0,000	0,000	0,005	0,010

	ANOVA p-vaules					
	Treatment (T)	**Genotype (G)**	**Day (D)**	**TxG**	**TxD**	**TxGxD**
ADP-Glucose (nmol/g)	n.s	n.s	0,016	n.s	0,001	n.s
3PGA (nmol/g)	0,000	0,002	0,005	n.s	0,000	n.s
PEP (nmol/g)	0,000	n.s	n.s	n.s	0,000	n.s
UDP-Glucose (nmol/g)	n.s	0,003	n.s	n.s	n.s	n.s
Trans-Aconitate (nmol/g)	0,000	n.s	0,001	n.s	0,000	n.s
Cis-Aconitate	n.s	0,013	n.s	n.s	n.s	n.s
Fru1,6bisP	0,001	0,000	0,000	n.s	0,000	0,000
UDP	n.s	n.s	n.s	n.s	n.s	n.s
Malate (µmol/g)	n.s	n.s	n.s	0,000	0,000	n.s
Citrate (µmol/g)	0,000	n.s	0,000	0,000	0,000	n.s
Isocitrate (nmol/g)	0,000	0,000	0,000	0,001	0,001	0,025
Glucose (nmol/g)	0,000	0,000	0,000	0,002	n.s	0,015
Fructose (nmol/g)	0,000	0,000	0,000	0,000	0,000	0,000
Saccharose (nmol/g)	n.s	n.s	0,000	n.s	0,000	n.s
Inositol (nmol/g)	n.s	0,022	n.s	0,006	0,023	n.s
Glycerol (nmol/g)	0,001	0,000	0,000	n.s	0,015	0,000
Erythritol (nmol/g)	0,000	0,003	0,000	0,001	0,000	0,042
Trehalose (nmol/g)	0,042	0,000	0,003	0,000	0,000	n.s
Galactose (nmol/g)	0,000	0,000	0,000	0,001	0,000	0,000
Rhamnose (nmol/g)	0,000	0,003	0,001	0,000	0,000	n.s
Gene expression data						
Mannose-6-phosphate isomerase	0,001	0,000	n.s	0,000	0,000	0,017
GDP Mannose pyrophosphorylase	n.s	0,000	n.s	n.s	n.s	
GDP Mannose pyrophosphorylase	n.s	0,000	0,005	n.s	0,016	0,005
GDP Mannose pyrophosphorylase	0,042	n.s.	0,019	n.s	n.s	n.s
GDP-mannose 3,5-epimerase	n.s	n.s.	0,000	n.s	0,000	n.s
GDP-mannose 3,5-epimerase	n.s	0,000	0,000	n.s	0,000	0,009
VTC2 (GDP-L-galactose phosphorylase)	n.s	0,014	0,000	n.s	n.s	n.s
GDP-L-galactose phosphorylase	n.s	0,031	0,041	n.s	n.s	n.s
L-galactose-1P- phosphatase	0,009	0,000	0,001	n.s	n.s	0,000
L-galactose dehydrogenase	0,001	0,000	n.s	0,011	n.s	0,009
L-galactono-1,4-lactone dehydrogenase	n.s	0,000	0,000	0,010	n.s	0,002
L-galactono-1,4-lactone dehydrogenase	n.s	n.s	0,045		n.s	n.s
***Myo*-inositol oxygenase**	0,000	0,000	n.s	0,000	n.s	0,001

Chapter 2

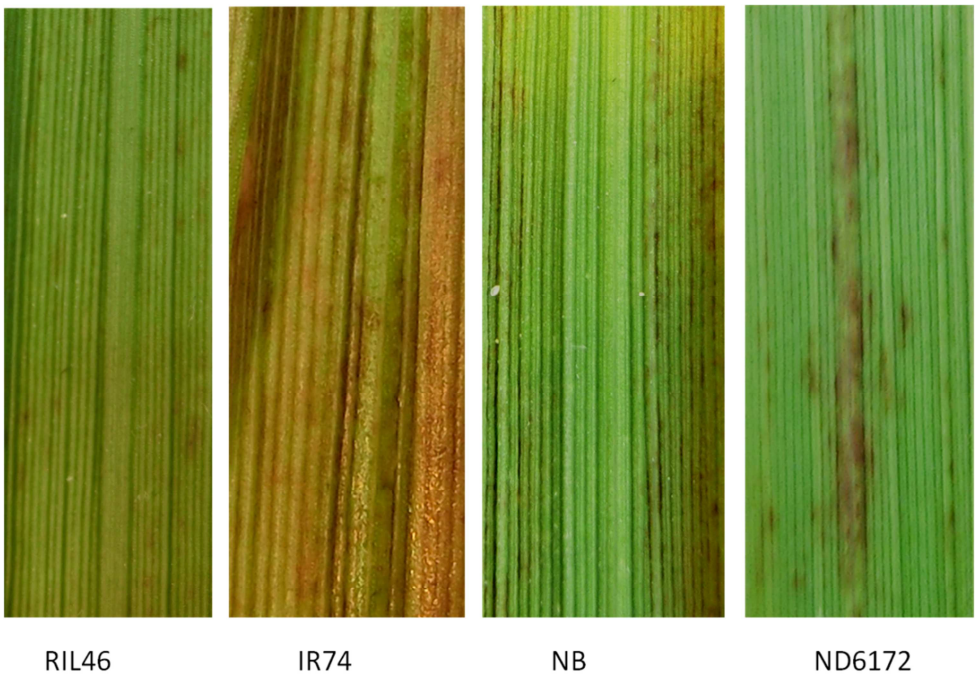


Fig S1: Leaf bronzing symptoms of RIL46/IR74 and NB/ND6172 21 days after –Zn treatment. Leaf bronzing served as in indicator of oxidative stress.

Chapter 3

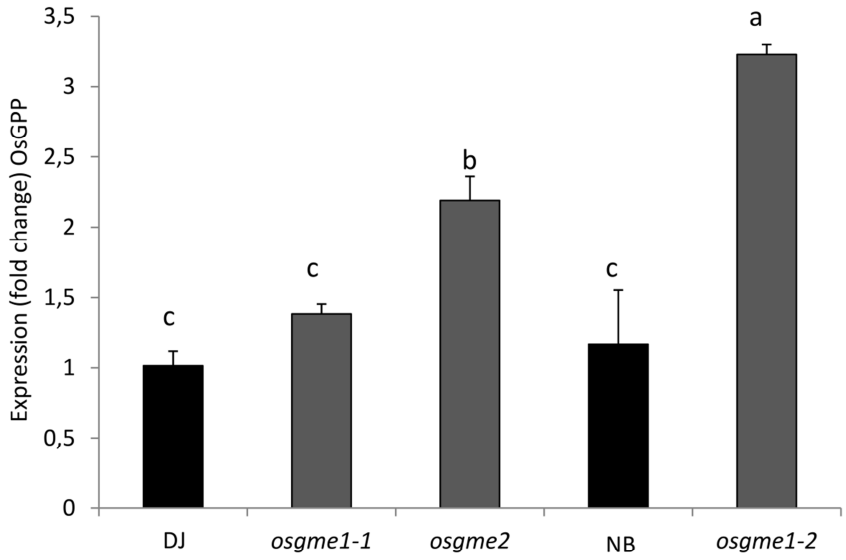
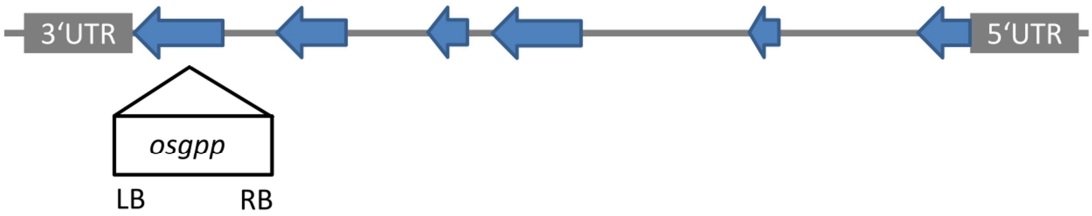
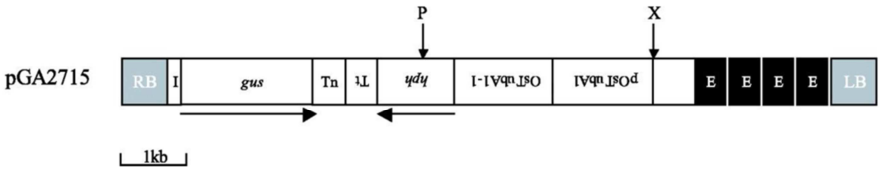


Fig. S1: Expression level of *OsGGP* in the *OsGME* mutant lines and their corresponding wild-types. Expression of *OsGME* is normalized by the expression of the internal reference gene Os05g0564200 (RAP-ID, annotated as U2 snRNP). Expression levels of the mutant lines are expressed as fold-changes relative to their corresponding wild-types. Data bars not sharing the same superscript letter are statistically different at $P < 0.05$ by LSD-test. Vertical bars indicate standard errors (n=4).

OsGPP

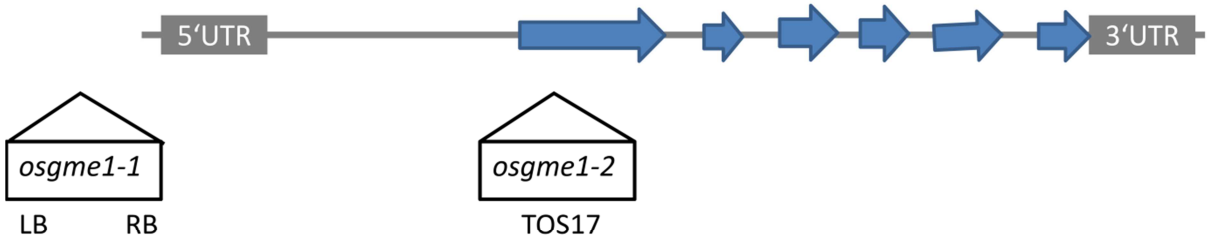


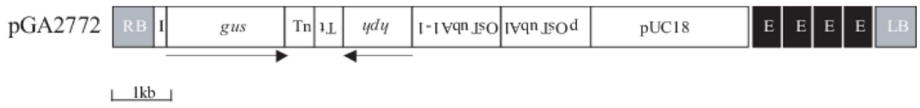


RAB locus ID: Os12g0190000
MSU locus ID: Os12g08810

Jeon et al. (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561-70.

OsGME1

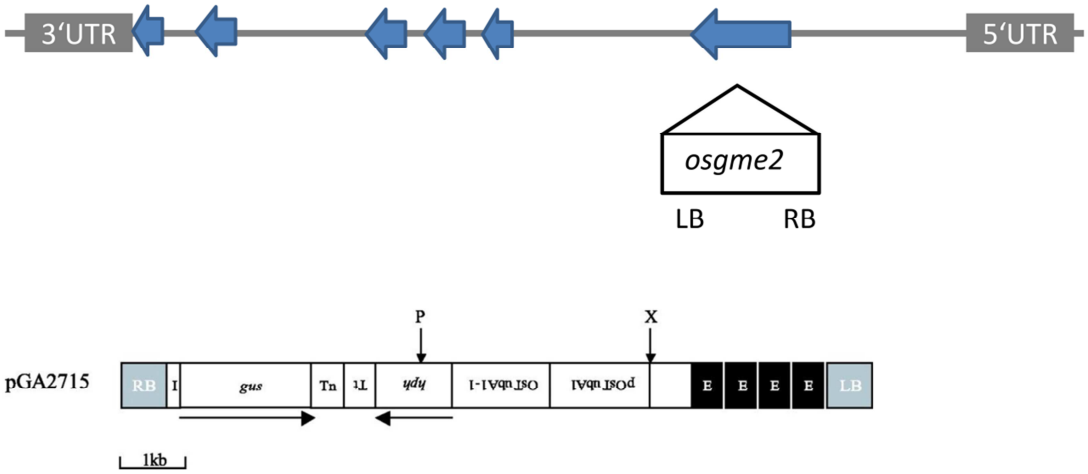




RAB locus ID: Os10g0417600
MSU locus ID: Os10g28200

Jeong et al. (2006) Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. Plant J 45:123-32.

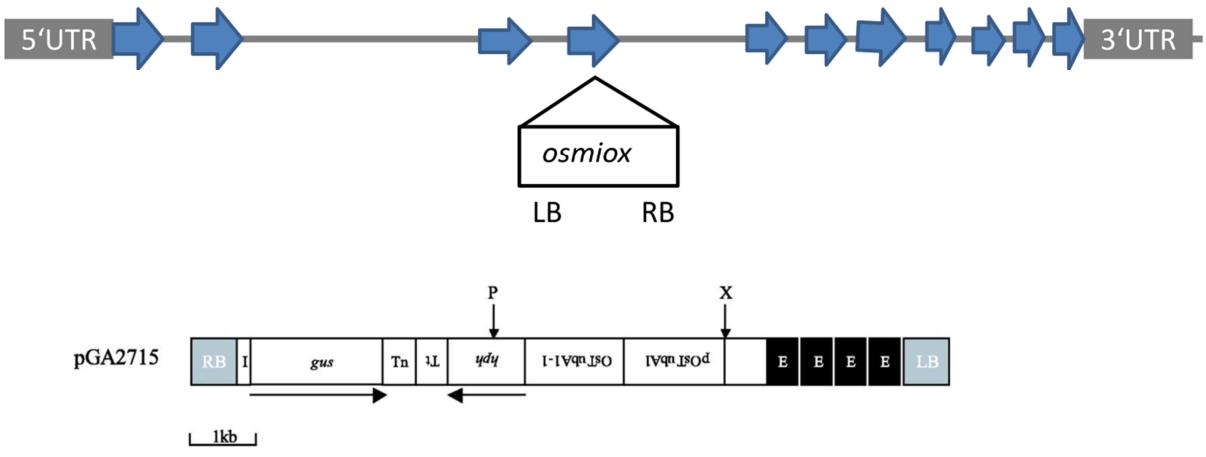
OsGME2



RAB locus ID: Os11g0591100
MSU locus ID: Os11g37890

Jeon et al. (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561-70.

OsMIOX



RAB locus ID: Os06g0561000
MSU locus ID: Os06g36560

Jeon et al. (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561-70.

Fig. S2: Genetic structure of the mutant lines. Blue arrows indicate exons. The place of insertion and direction of the T-DNA is displayed as well as the used vector.

Table S1: Primers used for identification of homozygous plants (a) and gene expression analysis (b)

a) **Primer genotyping**

	forward	**reverse**
osgme1-1	CATAATGGGTGTGTTCGGTG	CGTGAAAAGTTAACAGCGACG
osgme2	TCTTCCTTGATCCCCAACAG	ACCTCTTGAACGGTTTGTCG
osggp	TCCAACTACCAGGAGGATGC	GGACTCGCCTTGAAAGCATA
osmiox	ATTTTGCTGACCAAAGTGGC	CGTATGGCCTACCTGCTAGTG
T-DNA right border	AACGCTGATCAATTCCACAG	

b) **Primer geneexpression**

	forward	**reverse**
osgme1-1	GCCATGAGTTCCACCTTGTT	GCATGCACTTGAGGCATAGA
osgme2	GATGCCTATGGCTTGGAAAA	CAAAACGGTCAGTGGAGGTT
osggp	AGTGCCTTTCCCTGTTGAGA	ATACAAGCGCTGGAAACCAC
osmiox	ACCCAAAGCTCAACACCAAGT	CCTTGTCCTCATCGTTCATCA
osgme1-2	GCCATGAGTTCCACCTTGTT	GCATGCACTTGAGGCATAGA

Table S2: Concentration of metabolites, enzyme activities and photosynthetic parameters of the mutant lines. Significant differences were determined by LSD-test with the following significance levels: 0.05, *, 0.01, **, 0.001, *** (n=4).

	DJ	*osggp*		*osmiox*	
Glucuronic acid nmol g^{-1} FW	18,86	9,38	ns	8,75	ns
Glucose-1-P nmol g-1 FW	2,45	2,40	ns	1,90	ns
Trehalose-6-P nmol g-1 FW	31,43	27,96	ns	20,18	ns
Sucrose-6-P nmol g-1 FW	7,02	5,63	ns	6,41	ns
Succinate nmol g-1 FW	60,66	58,56	***	66,10	ns
Malate nmol g-1 FW	5210,40	4433,66	ns	3178,14	*
Pyruvate nmol g-1 FW	46,92	33,81	ns	45,51	ns
Oxoglutarate nmol g-1 FW	417,73	360,13	**	354,42	*
Fumarate nmol g-1 FW	8,72	11,57	ns	7,07	ns
Ribose-5-P nmol g-1 FW	19,09	12,87	*	17,14	ns
AMP nmol g-1 FW	56,16	41,46	ns	14,42	*
ADPGlc nmol g-1 FW	2,85	1,53	ns	0,58	ns
3PGA nmol g-1 FW	609,66	622,38	*	550,43	ns
Citrate nmol g-1 FW	4654,42	5463,12	**	2268,75	**
Isocitrate nmol g-1 FW	812,22	1294,94	***	657,30	ns
Trans aconitate nmol g-1 FW	33,67	31,14	ns	21,84	ns
PEP nmol g-1 FW	262,76	243,46	ns	282,05	ns
UPP-Glc nmol g-1 FW	109,42	111,63	ns	68,29	ns
Cis aconitate nmol g-1 FW	14,75	8,51	ns	7,34	ns
ADP nmol g-1 FW	19,80	22,04	ns	18,12	ns
Fru-1,6-bis-P nmol g-1 FW	1,54	2,22	ns	1,74	ns
UDP nmol g-1 FW	13,38	9,54	ns	12,72	ns
ATP nmol g-1 FW	14,39	13,71	ns	11,74	ns
Hexose-P nmol g-1 FW	80,99	72,18	ns	50,30	ns
Mannose-1-P nmol g-1 FW	681,20	681,11	ns	462,16	ns
Gulono lactone nmol g-1 FW	366,65	380,13	ns	329,36	ns
Threonic acid nmol g-1 FW	1958,07	916,17	***	1778,16	ns
Tartaric acid nmol g-1 FW	11,34	7,66	ns	10,34	ns
Oxalate nmol g-1 FW	5949,40	4671,72	***	5736,44	ns
GDP-mannose nmol g-1 FW	3,50	4,58	*	2,79	ns
Inositol nmol g-1 FW	684,03	587,13	*	658,34	ns
Glycerol nmol g-1 FW	14,98	21,44	ns	26,76	ns
Erythritol nmol g-1 FW	111,98	115,46	ns	132,87	ns
Galactose nmol g-1 FW	70,54	48,44	ns	109,29	*
Glucose µmol g-1 FW	1,16	0,42	ns	0,77	ns

	DJ	*osggp*		*osmiox*	
Fructose µmol g-1 FW	1,49	0,53	ns	0,94	ns
Sucrose µmol g-1 FW	14,25	11,10	ns	10,86	ns
Ser nmol g-1 FW	1055,21	740,05	***	1485,22	ns
Gln nmol g-1 FW	1007,26	558,06	ns	499,14	ns
Gly nmol g-1 FW	220,60	167,52	ns	224,69	ns
Asp nmol g-1 FW	842,81	797,46	*	802,09	ns
Glu nmol g-1 FW	1905,63	1853,02	ns	1842,98	ns
Thr nmol g-1 FW	380,34	303,48	ns	518,04	ns
Ala nmol g-1 FW	807,31	610,01	**	1059,14	ns
GABA nmol g-1 FW	52,09	39,59	*	66,38	ns
Pro nmol g-1 FW	60,43	54,43	ns	77,71	**
Lys nmol g-1 FW	59,53	49,49	ns	100,23	*
Tyr nmol g-1 FW	330,86	246,62	ns	373,81	ns
Val nmol g-1 FW	131,17	122,43	ns	213,84	**
Ile nmol g-1 FW	53,65	52,41	ns	103,91	***
Leu nmol g-1 FW	54,86	49,64	ns	96,54	**
Phe nmol g-1 FW	54,18	58,93	*	99,51	***
APX µmol mg^{-1} min^{-1}		0,80	**	1,37	**
DHAR µmol mg^{-1} min^{-1}		0,12	**	0,15	ns
MDHAR µmol mg^{-1} min^{-1}		35,76	***	52,96	ns
GR nmol mg^{-1} min^{-1}		7,68	ns	16,15	ns
A (mmol m^{-2} s^{-1})		8,75	***	12,15	**
g (mol m-2 s-1)		0,15	ns	0,16	ns
ΦPSII		0,31	**	0,45	ns
Fv′/Fm′		0,63	ns	0,70	ns
qP		0,50	ns	0,63	ns
qN		0,32	ns	0,30	ns
NPQ		0,30	ns	0,33	ns
V_{max} (µmol m^{-2} s^{-1})		45,23	***	59,60	**
J_{max} (µmol m^{-2} s^{-1})		115,43	***	128,79	ns

	DJ-2	*osgme1-1*		*osgme2*	
Glucuronic acid nmol g^{-1} FW	14,12	14,53	ns	12,26	ns
Glucose-1-P nmol g-1 FW	2,60	2,36	ns	2,99	ns
Trehalose-6-P nmol g-1 FW	19,39	14,96	ns	28,11	ns
Sucrose-6-P nmol g-1 FW	6,45	6,34	ns	7,11	ns
Succinate nmol g-1 FW	68,63	52,65	*	57,44	ns
Malate nmol g-1 FW	4067,37	4415,65	*	4991,85	*
Pyruvate nmol g-1 FW	55,06	47,89	ns	48,98	ns
Oxoglutarate nmol g-1 FW	373,08	321,30	ns	309,38	ns
Fumarate nmol g-1 FW	7,94	7,67	ns	11,43	ns
Ribose-5-P nmol g-1 FW	19,35	12,96	ns	23,95	ns
AMP nmol g-1 FW	72,77	37,25	ns	50,80	ns
ADPGlc nmol g-1 FW	2,05	1,10	ns	2,03	ns
3PGA nmol g-1 FW	590,44	556,25	ns	609,30	ns
Citrate nmol g-1 FW	3799,21	4543,20	ns	3754,51	ns
Isocitrate nmol g-1 FW	881,72	758,40	ns	936,59	ns
Trans aconitate nmol g-1 FW	31,76	30,35	ns	30,98	ns
PEP nmol g-1 FW	265,80	264,81	ns	287,28	ns
UPP-Glc nmol g-1 FW	92,06	87,13	ns	92,22	ns
Cis aconitate nmol g-1 FW	8,85	9,51	ns	13,89	ns
ADP nmol g-1 FW	14,37	18,40	ns	19,24	ns
Fru-1,6-bis-P nmol g-1 FW	1,86	6,21	ns	1,76	ns
UDP nmol g-1 FW	13,84	12,54	ns	10,32	ns
ATP nmol g-1 FW	11,82	10,93	ns	16,44	*
Hexose-P nmol g-1 FW	85,47	70,25	ns	70,82	ns
Mannose-1-P nmol g-1 FW	547,23	547,39	ns	544,32	ns
Gulono lactone nmol g-1 FW	430,65	399,49	ns	490,94	*
Threonic acid nmol g-1 FW	1917,67	1960,74	ns	1627,25	ns
Tartaric acid nmol g-1 FW	11,49	11,34	ns	13,08	ns
Oxalate nmol g-1 FW	5482,71	4931,63	ns	5381,21	ns
GDP-mannose nmol g-1 FW	2,84	2,75	ns	3,66	ns
Inositol nmol g-1 FW	690,23	771,53	ns	743,65	ns
Glycerol nmol g-1 FW	39,11	27,60	ns	28,31	ns
Erythritol nmol g-1 FW	123,53	109,23	ns	118,98	ns
Galactose nmol g-1 FW	100,58	129,24	ns	49,33	ns
Glucose µmol g-1 FW	0,82	0,66	ns	1,01	ns
Fructose µmol g-1 FW	0,96	1,04	**	1,15	**
Sucrose µmol g-1 FW	12,18	13,93	ns	13,62	ns
His nmol g-1 FW	37,28	50,36	ns	40,07	ns
Asn nmol g-1 FW	900,78	2814,50	**	915,04	*
Ser nmol g-1 FW	836,73	1550,14	ns	716,67	ns
Gln nmol g-1 FW	693,11	1367,69	ns	506,15	ns
Gly nmol g-1 FW	230,60	236,81	ns	155,34	ns
Asp nmol g-1 FW	763,11	822,97	ns	746,71	ns

	DJ-2	*osgme1-1*		*osgme2*	
Glu nmol g-1 FW	1668,98	1655,48	ns	1757,31	ns
Thr nmol g-1 FW	374,49	486,26	ns	279,71	ns
Ala nmol g-1 FW	886,91	1080,07	ns	623,09	ns
GABA nmol g-1 FW	66,69	54,96	ns	64,41	ns
Pro nmol g-1 FW	53,69	71,45	*	55,40	ns
Lys nmol g-1 FW	57,91	62,13	ns	44,43	ns
Tyr nmol g-1 FW	295,22	455,61	ns	376,04	ns
Val nmol g-1 FW	137,93	160,66	ns	122,00	*
Ile nmol g-1 FW	52,89	53,70	ns	50,39	ns
Leu nmol g-1 FW	50,11	50,74	ns	48,54	ns
Phe nmol g-1 FW	59,36	57,16	ns	61,73	ns
APX µmol mg^{-1} min^{-1}	2,38	1,37	**	1,47	**
DHAR µmol mg^{-1} min^{-1}	0,18	0,13	*	0,14	*
MDHAR µmol mg^{-1} min^{-1}	60,88	51,77	ns	51,88	ns
GR nmol mg^{-1} min^{-1}	14,02	13,65	ns	14,75	ns
A (mmol m^{-2} s^{-1})	17,78	11,80	**	17,37	ns
g (mol m-2 s-1)	0,17	0,15	ns	0,18	ns
ΦPSII	0,45	0,31	*	0,31	*
Fv′/Fm′	0,66	0,51	ns	0,62	ns
qP	0,68	0,57	ns	0,49	ns
qN	0,27	0,60	**	0,55	**
NPQ	0,28	1,08	ns	0,80	ns
V_{max} (µmol m^{-2} s^{-1})	77,39	62,36	*	71,07	ns
J_{max} (µmol m^{-2} s^{-1})	144,75	127,49	ns	130,14	ns

	NB	*osgme1-2*	
Glucuronic acid nmol g^{-1} FW	12,40	12,48	ns
Glucose-1-P nmol g-1 FW	2,38	3,21	ns
Trehalose-6-P nmol g-1 FW	18,60	19,96	ns
Sucrose-6-P nmol g-1 FW	5,00	6,24	ns
Succinate nmol g-1 FW	93,81	65,24	**
Malate nmol g-1 FW	2966,73	4307,02	**
Pyruvate nmol g-1 FW	83,97	43,21	**
Oxoglutarate nmol g-1 FW	339,96	376,93	ns
Fumarate nmol g-1 FW	7,95	7,35	ns
Ribose-5-P nmol g-1 FW	22,89	21,60	ns
AMP nmol g-1 FW	53,20	34,33	ns
ADPGlc nmol g-1 FW	1,52	1,50	ns
3PGA nmol g-1 FW	499,46	712,56	*
Citrate nmol g-1 FW	3357,09	4293,18	ns
Isocitrate nmol g-1 FW	1044,09	807,79	ns
Trans aconitate nmol g-1 FW	35,23	30,41	ns
PEP nmol g-1 FW	226,27	329,96	*
UPP-Glc nmol g-1 FW	77,87	94,17	ns
Cis aconitate nmol g-1 FW	9,10	7,85	ns
ADP nmol g-1 FW	13,24	20,05	ns
Fru-1,6-bis-P nmol g-1 FW	3,57	5,61	ns
UDP nmol g-1 FW	12,30	13,22	ns
ATP nmol g-1 FW	8,78	13,68	ns
Hexose-P nmol g-1 FW	78,64	91,75	ns
Mannose-1-P nmol g-1 FW	488,40	720,41	**
Gulono lactone nmol g-1 FW	533,25	713,84	*
Threonic acid nmol g-1 FW	1784,70	1198,64	**
Tartaric acid nmol g-1 FW	10,65	14,05	ns
Oxalate nmol g-1 FW	4721,72	5623,57	ns
GDP-mannose nmol g-1 FW	2,45	4,22	**
Inositol nmol g-1 FW	775,23	738,59	ns
Glycerol nmol g-1 FW	44,56	36,49	ns
Erythritol nmol g-1 FW	110,81	123,96	ns
Galactose nmol g-1 FW	91,16	86,41	ns
Glucose µmol g-1 FW	0,52	0,70	ns
Fructose µmol g-1 FW	0,78	0,82	**
Sucrose µmol g-1 FW	10,54	14,67	**
His nmol g-1 FW	58,79	41,01	**
Asn nmol g-1 FW	1679,55	1554,65	ns
Ser nmol g-1 FW	1235,05	1049,18	ns
Gln nmol g-1 FW	1099,50	1038,53	ns
Gly nmol g-1 FW	238,74	234,71	*
Asp nmol g-1 FW	802,92	810,64	ns

	NB	*osgme1-2*	
Glu nmol g-1 FW	1783,83	1962,98	ns
Thr nmol g-1 FW	423,62	371,94	ns
Ala nmol g-1 FW	860,69	719,59	ns
GABA nmol g-1 FW	85,89	51,23	ns
Pro nmol g-1 FW	62,50	53,83	*
Lys nmol g-1 FW	76,36	53,76	ns
Tyr nmol g-1 FW	440,04	273,58	ns
Val nmol g-1 FW	155,19	127,78	ns
Ile nmol g-1 FW	48,78	37,46	ns
Leu nmol g-1 FW	49,08	38,36	ns
Phe nmol g-1 FW	61,98	42,24	ns
APX µmol mg^{-1} min^{-1}	1,17	1,31	ns
DHAR µmol mg^{-1} min^{-1}	0,15	0,14	ns
MDHAR µmol mg^{-1} min^{-1}	49,79	49,63	ns
GR nmol mg^{-1} min^{-1}	13,38	16,04	ns
A (mmol m^{-2} s^{-1})	16,14	13,44	*
g (mol m-2 s-1)	0,20	0,16	ns
ΦPSII	0,36	0,20	*
Fv′/Fm′	0,57	0,63	**
qP	0,85	0,31	ns
qN	0,50	0,55	ns
NPQ	1,29	0,88	ns
V_{max} (µmol m^{-2} s^{-1})	78,51	63,68	*
J_{max} (µmol m^{-2} s^{-1})	150,22	138,09	*