Institut für Nutzpflanzenwissenschaften und Ressourcenschutz

- Pflanzenernährung -

# Genetic and Physiological Analyses of the Tolerance Mechanisms to Ferrous Iron Toxicity in Rice (*Oryza sativa* L.)

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# Dedication

This thesis is dedicated to my grandmother (21.10.1924 – 25.04.2015) who loves and inspires me all the way of my life and study.

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# List of abbreviations

| | |
|---|---|
| AO | Ascorbate oxidase |
| AsA | Ascorbic acid |
| Cd | Cadium |
| CSSL | Chromosome segment substitution line |
| Cu | Copper |
| DHA | Dehydroascorbic acid |
| DHAR | Dehydroascorbate reductase |
| DPD | 2,2'-bipyridyl |
| DTT | Dithiothreitol |
| Fe | Iron |
| GLM | General linear model |
| GO | Gene otonlogy |
| GST | Glutathione-S-transferase |
| GWAS | Genome-wide associaiton study |
| IDA | Iron deficiency anemia |
| IRGSP | International rice genome sequencing project |
| LBS | Leaf bronzing score |
| LD | Linkage disequlibrium |
| MAF | Minor allele frequency |
| MAs | Mugineic acid family phytosiderophores |
| MDA | Malondialdehyde |
| MDHA | Monodehydroascorbic acid |
| MDHAR | Monodehydroascorbate reductase |
| MLM | Mixed linear model |
| Mn | Manganese |
| NA | Nicotianamine |
| Pb | Lead |
| PCA | Pricinple component analysis |
| POX | Unspecific peroxidase |
| qq-plot | Quanile-quantile plot |
| QTL | Quantitative trait locus |
| RIL | Recombinant inbred line |
| ROS | Reactive oxygen species |
| SNP | Single nucleotide polymorphism |

| | |
|---|---|
| t-LBS | Squared-root transformed leaf bronzing score |
| WHO | World health organization |
| Zn | Zinc |

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# Abstract

Rice is a widely consumed staple food for more than half of the world population. Iron (Fe) toxicity is a major nutrient disorder affecting rice production. The understanding of the genetic and physiological basis of Fe tolerance mechanisms can provide useful information for the breeding of tolerant varieties. This thesis is structured into three major parts: (I) Quantitative trait locus (QTL) mapping using two bi-parental populations exposed to an acute Fe stress (1,000 ppm $Fe^{2+}$ for 5 days). QTLs were detected on several chromosomes (1, 2, 3, 4, 7, 8 and 12) indicating a complex genetic structure for Fe toxicity tolerance. Some QTLs were co-localized with previously reported QTLs on chromosome 1 and 2. One tolerant recombinant inbred line (RIL) FL510 showing an Fe exclusion mechanism was investigated along with two parental lines regarding root oxidizing power. It was found that the root architecture favored the root oxidizing ability in FL510. (II) In a second chapter, a tolerant RIL carrying a shoot-based tolerance mechanism was subjected to in-depth physiological analysis comprising both transcriptomic and biochemical approaches. Three hypotheses were tested to elucidate the roles of (1) Fe uptake, transport and partitioning, (2) biosynthesis of antioxidants and (3) antioxidant enzymes in shoot-based tolerance. It was found that the tolerance in FL483 was conferred by relatively lower ascorbate redox state controlled by dehydroascorbate reductase and ascorbate oxidase activity. A pro-oxidant activity of ascorbate was demonstrated *in planta* for the first time. (III) In a third chapter, a genome-wide association study (GWAS) was employed to investigate the genetic basis of Fe tolerance with a diverse panel consisting of 329 rice accessions genotyped by 44,100 single nucleotide polymorphism (SNP) markers. Among the different rice sub-populations, temperate *japonica* and aromatic showed more tolerance than *indica* and tropical *japonica* while *aus* showed intermediate tolerance. Two glutathione-S-transferase genes in one locus co-localized with previously detected QTLs on chromosome 1 for the trait of leaf bronzing score. Contrasting haplotypes at this locus showed sequence polymorphism in the two candidate genes. The tolerance underlying the locus was associated with low ascorbate redox state controlled by dehydroascorbate reductase in shoots. In summary, these efforts will contribute to the breeding of more adapted varieties and to a better understanding of Fe toxicity tolerance mechanisms in plants.

# Zusammenfassung

Reis (*Oryza sativa* L.) ist das wichtigste Grundnahrungsmittel für mehr als die Hälfte der Weltbevölkerung. Die übermäßige Aufnahme von Eisen (Fe) führt zu einem schwerwiegenden Nährstoffungleichgewicht, das den Reisanbau weltweit beeinträchtigen kann. Für die Züchtung von toleranten Reissorten ist es essentiell, die genetischen und physiologischen Toleranzmechanismen gegen Eisentoxizität zu verstehen. Die vorliegende Arbeit gliedert sich in drei Teile: (I) Bestimmung von quantitative trait locus (QTL) in einer biparentalen Population, die akutem Eisenstress ausgesetzt wurde (1,000 ppm $Fe^{2+}$ für 5 Tage). QTLs wurden auf etlichen Chromosomen entdeckt (1, 2, 3, 4, 7, 8 und 12), was auf eine komplexe genetische Struktur des Toleranzmechanismus hindeutet. Einige der QTLs co-lokalisierten mit bereits beschriebenen QTLs auf den Chromosomen 1 und 2. Die tolerante rekombinante Inzuchtlinie (RIL) FL510 zeichnete sich durch einen Wurzel-basierten Fe Exklusionsmechanismus aus, woraufhin die Oxidationskraft der Wurzeln untersucht wurde. Dabei zeigte sich, dass die Wurzelarchitektur der Linie FL510 deren Oxidationskraft begünstigte. (II) Im zweiten Kapitel wurde eine RIL mit Spross-basiertem Toleranzmechanismus mit Hilfe von transkriptionellen und biochemischen Analysen eingehend physiologisch charakterisiert. Die Rolle von (1) Eisenaufnahme, -transport und –verteilung; (2) Biosynthese von Antioxidantien und (3) antioxidativen Enzymen in Spross-basierten Toleranzmechanismen wurde untersucht. Es konnte gezeigt werden, dass die Toleranz der Linie FL483 gegenüber Eisenstress auf einem niedrigeren Ascorbat-Redox-Status basiert, der durch die Enzymaktivität der Dehydroascorbatreduktase und Ascorbatoxidase reguliert wird. Hierbei wurde erstmalig *in planta* eine prooxidative Aktivität von Ascorbat nachgewiesen. (III) Im dritten Kapitel wurde eine genomweite Assoziationsstudie (GWAS) durchgeführt um die genetische Grundlage von Eisen Toleranz in einem Panel von 329 diversen Reislinien zu untersuchen, die mit jeweils 44,100 Einzelnukleotid-Polymorphismen (englisch: single nucleotide polymorphisms, SNPs) genotypisiert sind. Dabei waren die temperate *japonica* und *aromatischen* Subpopulationen toleranter als *indica* und tropische *japonica*, während *aus* intermediär tolerant war. Das typische Verfärben der Blätter bei Fe Stress (“leaf bronzing”) wurde mit zwei Glutathion-S-Transferase Genen assoziiert, die mit einem bereits beschriebenen QTL auf Chromosom 1 co-lokalisierten. Gegensätzliche Haplotypen für diesen Locus zeigten Sequenzunterschiede innerhalb der beiden Kandidatengene. Damit wurde der niedrigere Ascorbat-Redox-Status aufgrund von veränderter Dehydroascorbatreduktase Aktivität im Spross in Zusammenhang gebracht. Diese Arbeit trägt zum grundlegenden Verständnis von Fe Toleranzmechanismen in Pflanzen bei sowie zur erfolgreichen Züchtung von besser angepassten Reissorten bei.

# Chapter I. Research Background

## 1.1 Food security and rice production

Due to the continuously growing population, decreasing arable lands and water pollution, we face the challenges to feed more than 9 billion people in the middle of 21st century (FAOSTAT, http://faostat3.fao.org/browse/O/*/E). Especially in developing countries, which are mostly located in Asia and Africa, food security is a big issue to be solved. Even though marked growth of food production was achieved within the past half-century, around 795 million people today are still undernourished and even more suffer from some form of micronutrient malnourishment (FAO 2015).

Among the staple foods, rice (genus *Oryza*) is the most important crop in the developing world and consumed by more than half of the world population (Seck et al. 2012). It provides the predominant dietary energy source for 17 countries in Asia and the Pacific, 9 countries in North and South America and 8 countries in Africa (FAO 2005). Compared with other staple foods (e.g. maize, wheat and potato), rice (brown) provides the most energy (1549 KJ/100 g), manganese (143 mg/100 g), phosphorus (333 mg/100 g) and vitamin B5 (1.49 mg/100 g) (USDA 2014).

Many efforts were made to improve the rice production since the 1960s. For example, the breeding of high-yield and semi-dwarf variety IR8 in 1966, so-called green revolution in rice, markedly increased the rice production in the Philippines from 3.7 to 7.7 million tons within two decades (Sasaki et al. 2002; FAO 2011). The application of fertilizers and pesticides, integrated water and soil managements and elite rice varieties contributed to the marked increase of the rice production since the 1960s (Tilman et al. 2002). From 1961 to 2013, the rice production in the world was increased about 3.4 folds (Fig. 1, FAOSTAT 2013). Still, it is estimated that we need 70% to 100% more food to feed the 9 billion people in the middle of this century (Godfray et al. 2010).

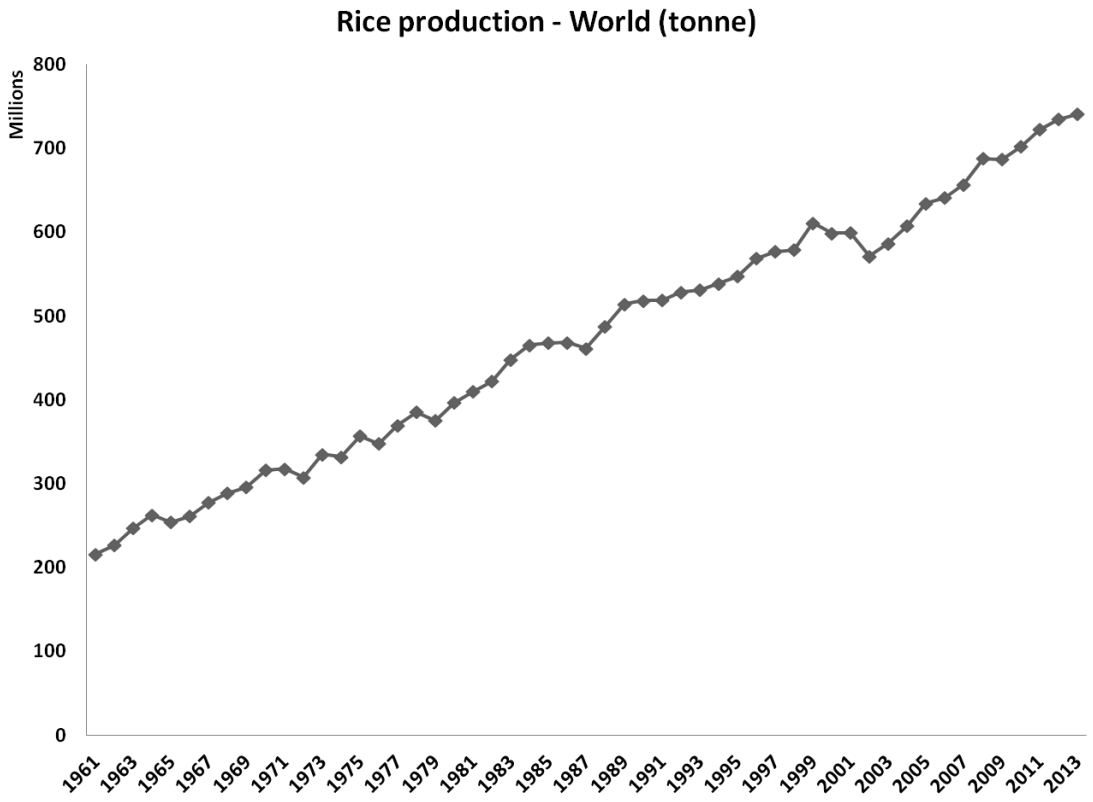



Figure 1. The production of rice in the world from 1961 to 2013 (data obtained from FAOSTAT 2013).

## 1.2 Rice species and domestication

There are two domesticated rice species in the world, Asian rice (*Oryza sativa* L.) and African rice (*Oryza glaberrima* Steud.) (Semon et al. 2005; Molina et al. 2011).

*Oryza sativa* was domesticated from one or both of two closely related species, *Oryza nivara* and *Oryza rufipogon* (Li et al. 2006). It is well accepted that the Asian rice was firstly domesticated in Yangtze River area in ancient China around 9000 years ago based on archaeological and genetic evidence (Gross and Zhao 2014; Molina et al. 2011). Several traits were considered as important during domestication process, such as grain shattering (Konishi et al. 2006; Li et al. 2006; Lin et al. 2007), grain pericarp color (Sweeney et al. 2006), glutinous grains (Wang et al. 1995; Bligh et al. 1998; Yamanaka et al. 2004), grain size/shape (Fan et al. 2006), grain fragrance/flavor (Bradbury et al. 2005), grain number (Ashikari et al. 2005) and grain weight/width (Song et al. 2007). There are two main sub-species in Asian rice, *japonica* and *indica*. Strong genetic differentiations were observed between these two sub-species suggesting the separated domestication and different origin parts from wild rice (Molina et al. 2011). Meanwhile, introgression of favorable alleles from

*japonica* was observed in *indica* (Feltus et al. 2004). However, the connection between the favorable alleles' introgression and the origin of *indica* and *japonica* still remain unclear. The possible scenarios for the origin of *japonica* and *indica* from *Oryza rufipogon* population were proposed by Gross and Zhao (2014) (Fig. 2).

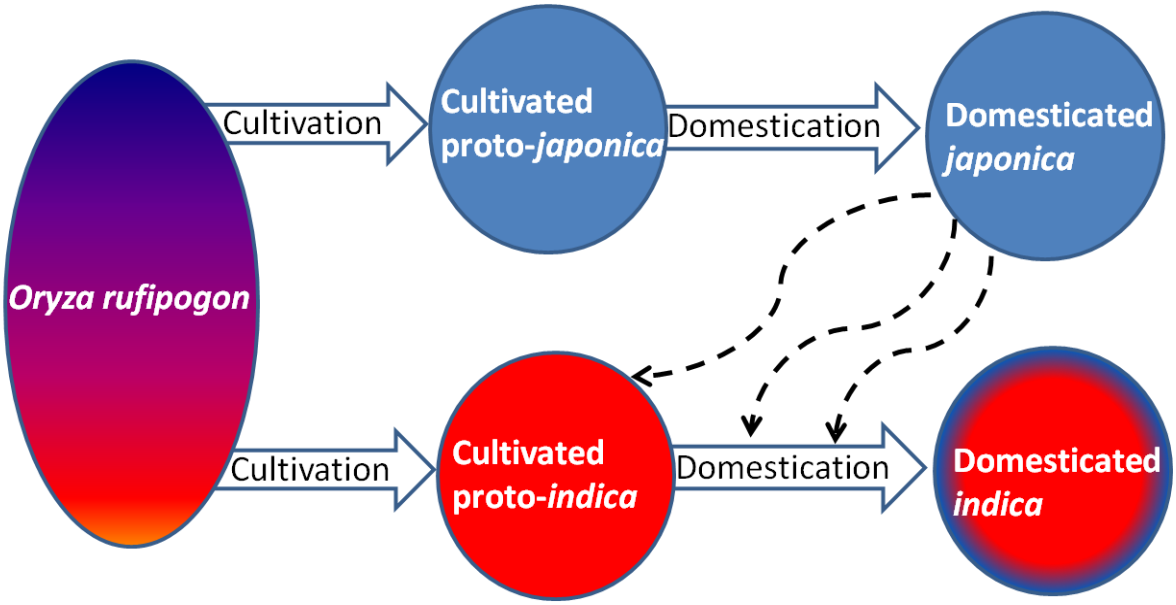



Figure 2. Possible scenarios for the origin of japonica and indica from a genetically diverse Oryza rufipogon population. Different colors represent different genetic background. Possible timing for the movement of domestication alleles from *japonica* into *indica* via hybridization is shown with dashed lines. Colors in the final domesticated *indica* circle represent the contributions from the original *Oryza rufipogon* populations (red) and introgression from *japonica* (blue) (Gross and Zhao 2014).

*Oryza glaberrima* was domesticated from the wild ancestor *Oryza barthii* in the Niger River delta about 3500 years ago (Semon et al. 2005; Linares 2002). Today, it is largely grown in West Africa. Compared to *Oryza sativa*, *Oryza glaberrima* shows only few morphological differences including relatively small pear-shaped grain with red bran, an olive-to-black seed-coat, straight panicles that are simply branched, and short, rounded ligules (Linares 2002). African rice varieties have certain negative features: the seed scatters easily, the grain is brittle and difficult to mill and, most importantly, the yields are lower. Despite the disadvantages, *Oryza glaberrima* plants have wide leaves that shade out weeds and show higher resistance to diseases and pests than *Oryza. sativa*. Moreover, African rice is tolerant to fluctuations in water depth, iron toxicity, infertile soils, severe climates and human neglect (Ellen and Fukui 1996; Linares 2002). In this study, we mainly focus on Asian rice.

## 1.3 The rice genome and its implications on crop research

Rice is a diploid plant with 12 chromosomes ranging from 23.2-43.2 Mb (http://rgp.dna.affrc.go.jp/IRGSP/). Because of its relatively small genome size (450 Mb) compared with other cereals, e.g. maize (2,300 Mb), wheat (17,100 Mb) or barley (5,500 Mb), and its importance as a food resource for mankind, rice has become an ideal model for genomic research in monocots (Morrell et al. 2012). To better understand the genome of rice, a *japonica* cultivar, Nipponbare was sequenced by clone-by-clone shotgun strategy in the International Rice Genome Sequencing Project (IRGSP) that was initiated in 1997. In December 2004, the IRGSP completed the sequencing of the rice genome (IRGSP 2005). Meanwhile, the Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/) and the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu/index.shtml) were created to provide manually curated annotations of rice genome (Ohyanagi et al. 2006; Ouyang et al. 2007). Up to date, 55,986 loci were identified including 39,045 non-transposable element (Non-TE) loci and 16,941 transposable element (TE) loci (RGAP 7). Among the non-TE loci, 49,066 gene models were identified and the average gene size is 2 853 bp with 4.9 exons and 3.9 introns per gene. A total of 17,272 gene models were found in TE loci and the average gene size is 3,223 bp with 4.2 exons and 3.2 introns per gene (http://rice.plantbiology.msu.edu/index.shtml). Another *indica* cultivar 93-11 was sequenced with whole-genome shotgun method by Beijing Genomics Institute (BGI, http://rise2.genomics.org.cn/page/rice/index.jsp) (Yu et al. 2002). It has 37,578 BGF genes with a genome size of 466 Mb (http://rice.genomics.org.cn/rice/jsp/chrview.jsp).

As the first completed and the best annotated genome for monocots and crops, rice offers invaluable information of the structure and function of genome for other crop plants (Liang et al. 2008). Comparing the gene structures and functions will enable us to identify the highly conserved or rapidly evolving regions among crop species, thus to elucidate plant genome evolution, speciation and domestication (IRGSP 2005).

## 1.4 Abiotic stresses in rice growth

Due to the integrated climate change and the sessile nature of plants, crop production losses caused by the undesirable environments have risen steadily within the past decades (Mickelbart et al. 2015). Many abiotic stresses, which refer to non-living factors that adversely affect plant cellular homeostasis and that ultimately impair the growth and fitness, occur in rice production (Vinocur and Altman 2005). Frequently observed abiotic stresses in rice production are water surplus (flooding),

water deficit (drought), temperature extremes, ion disorders (deficiency or toxicity) and ozone stress (Mickelbart et al. 2015). Abiotic stresses in rice fields exhibit complexities including the stress episodes, i.e. pulse stress (e.g. midday heat stress) and chronic stress (e.g. $Al^{3+}$ toxicity in soil), the timing of stress occurrence (e.g. vegetative and reproductive growth stage), multiple stresses (e.g. co-occurrence of salinity and Zn deficiency) and successive stresses (e.g. flooding followed by heat stress) (Gregorio et al. 2002; Jagadish et al. 2014; Li et al. 2014; Mickelbart et al. 2015).

To breed more environment adapted cultivars, the understandings of the genetic factors underlying the tolerance mechanisms to abiotic stress are crucial. Several tremendous achievements were obtained in the investigations of the tolerant loci and mechanisms to, for example flooding and drought stress. In Asian paddies, flooding is the most observed abiotic stress (Vallino et al. 2014). One Indian landrace carrying one locus, *SUBMERGENCE 1* (SUB1) conferring submergence tolerance by restricting underwater elongation growth through ethylene-promoted inhibition of gibberellins-mediated pathway, can survive more than 2 weeks of complete submergence (Xu et al. 2006). Another two genes, *SNORKEL1* and *SNORKEL2* were identified using quantitative trait loci (QTL) mapping combined with positional cloning. The expressions of these two genes were induced by the ethylene produced in plant under submergence conditions. The products of *SNORKEL1* and *SNORKEL2* then remarkably trigger the internode elongation through gibberellins-mediation (Hattori et al. 2009). One major QTL, *DEEPER ROOTING 1* (DRO1) was detected on chromosome 9 by (Uga et al. 2011) in a study about drought tolerance in rice. DRO1, which is involved in the root cell elongation, is negatively regulated by auxin and causes asymmetric root growth due to the downward bending of the root in response to gravity (Uga et al. 2013).

## 1.5 Physical, chemical and biological characteristics of iron

Iron (Latin *ferrum*, Fe) is a metal in the first transition series, which can rise to the cations with an incomplete *d* sub-shell of electrons (Balk and Schaedler 2014). Even though Fe listed in group 8 can exist in a range of oxidation states (from -2 to +6), ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) are the most common oxidation states. Different chemicals representing the oxidation states are shown in Figure 3. There are four stable isotopes of Fe occurring in nature including $^{54}Fe$ (5.845%), $^{56}Fe$ (91.754%), $^{57}Fe$ (2.119%) and $^{58}Fe$ (0.282%). These isotopes can be utilized in many aspects regarding medical practices; research purposes etc. (Beard et al. 2003; Dauphas and Rouxel 2006). Besides the various applications in industries, Fe is also an essential element involved in many vital processes for all living organisms except some

unusual bacterial species (Andrews 2000). Through the redox change between $Fe^{2+}$ and $Fe^{3+}$, Fe serves as an electron acceptor or donor (Brumbarova et al. 2015). This property makes Fe essential as a component of cytochromes, oxygen sensing, transporting and storing molecules (e.g. hemoglobin and myoglobin) and various enzymes (e.g. nitrate reductase for nitrogen fixation, superoxide dismutase and ribonucleotide reductase for DNA synthesis) (Briat and Lobréaux 1997; Andrews 2000; Ganz and Nemeth 2011).


Figure 3. Different forms of Fe chemicals including (A) pure Fe, (B) $FeSO_4 \cdot 7H_2O$ and (C) $FeCl_3$ are shown. The photo of (A) was obtained from Wikipedia (uploaded by Alchemist-hp), (B) from the website (http://www.arihantgroupindustries.com) and (C) taken by myself.

### 1.6 Fe uptake strategy in living organisms

To obtain adequate Fe for critical biological processes, living organisms evolved distinct approaches. In microorganisms, many bacteria and fungi secrete siderophores to generate soluble $Fe^{3+}$ complexes for efficient Fe uptake (Cornelis and Andrews 2010). The complexes are transported through membranes by specific transport systems (Stintzi et al. 2000). Another strategy, i.e. the reduction of $Fe^{3+}$ followed by the transport of $Fe^{2+}$ ions, was employed by *Legionella* spp, *Streptococcus* spp., *Saccharomyces cerevisiae* etc. (Guerinot 1994). Pathogenic bacteria (e.g. notorious *Yersinia pestis* and *Vibrio cholerae*) obtain Fe from heme, ferritin and transferrins in the host cells to fulfill their invasion, colonization and reproduction (Perry and Brubaker 1979; Stoebner and Payne 1988; Ratledge and Dover 2000).

Mammals obtain Fe exclusively from their diet. There are two basic forms of Fe in food stuff, inorganic Fe that is present in a variety of foods and cannot be efficiently absorbed and heme Fe that is mostly present in animal sources and can be efficiently absorbed (Andrews and Schmidt 2007). Fe absorption in humans takes place in the proximal small intestine, near the gastro-duodenal junction (Andrews and Schmidt

2007). Inorganic or non-heme Fe from the diet is firstly reduced by ferric-reductase (e.g. duodental cytochrome b) to $Fe^{2+}$ followed by the transport of $Fe^{2+}$ ions using divalent metal transporters (McKie et al. 2001). The transport of heme Fe from the gut lumen into enterocytes can be completed by heme carrier protein 1 (Shayeghi et al. 2005). After the decomposition of heme, released Fe is likely to be stored and transported in the same pathways as non-heme Fe (Andrews and Schmidt 2007). The absorption of Fe is regulated by at least three different aspects, which are recent dietary Fe uptake, total body Fe and the demand of Fe in erythropoiesis (Hahn et al. 1943; Finch 1994). Transferrin is an abundant, high-affinity Fe binding protein, which carries nearly all absorbed Fe in the serum except for very small amounts of Fe associated with albumin or other small molecules (Park et al. 1985).

Higher plants evolved two distinct strategies to absorb Fe from soil (Fig. 4) (Römheld and Marschner 1986). Most non-graminaceous plants employ Strategy I, which involves acidification of the rhizosphere through the secretion of protons (by $H^+$-ATPase), the reduction of $Fe^{3+}$ to $Fe^{2+}$ (by ferric-chelate reductase/oxidase) and the transport of $Fe^{2+}$ through plasma membrane using iron regulated transporter (IRT). Graminaceous plants including rice, maize and wheat, use Strategy II, which employs phytosiderophores (MAs) to chelate $Fe^{3+}$ and transport the $Fe^{3+}$-MAs complex into the cytosol using yellow-stripe-like (YSL) transporters. Although being classified as a Strategy II species, rice possesses iron-regulated transporters (*OsIRT*) that are novel Strategy I transporters.

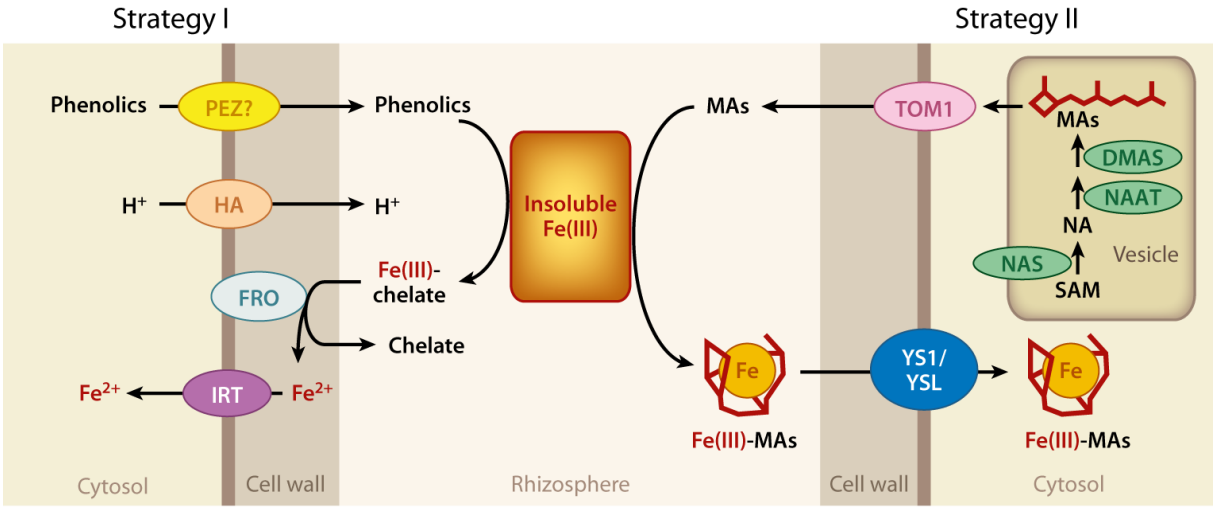



Figure 4. Fe uptake strategies in higher plants. In strategy I, prior to the reduction of $Fe^{3+}$ to $Fe^{2+}$, $Fe^{3+}$ ions are dissolved and mobilized through the cell wall. The generated $Fe^{2+}$ ions are transported by iron regulated transporters. In strategy II, insoluble $Fe^{3+}$ ions are chelated by mugenic acids forming soluble complexes followed by the transport of the complexes into cytosol. DMAS, deoxymugineic acid synthase; FRO, ferric-chelate reductase oxidase; HA, $H^+$-ATPase; IRT, iron-regulated transporter; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; PEZ, PHENOLICS EFFLUX ZERO; SAM, *S*-adenosyl-L-methionine; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1–like (Kobayashi and Nishizawa 2012).

## 1.7 Fe deficiency anemia and biofortification in rice

Due to the importance of Fe in biological processes, the human body has to tightly control Fe homeostasis (Wessling-Resnick 2010). Fe deficiency anemia (IDA) is the most common and widespread nutrient disorder in the world. The World Health Organization (WHO) estimates that more than 2 billion people are anemic due to Fe deficiency. Infants with IDA show significantly lower mental and psychomotor developmental index scores compared to those who are non-anemic (Walter et al. 1989). Severe anemia even leads to maternal and prenatal mortality (Allen 2000). In adults, IDA causes reduced work or exercise ability; impairs thermoregulation, the neurocognitive and immune system and disturbs glycemic index (Clark 2008). Additionally, IDA is associated with chronic kidney disease or congestive heart failure (Ezekowitz et al. 2003; McClellan et al. 2004). IDA is diagnosed by measuring the Fe concentration in hemoglobin and varies by sex and age. The most adopted standards are defined by the Centers for Disease Control and Prevention (CDC) in the U.S and the WHO (Killip et al. 2007). The primary prevention of IDA in infants are to breastfeed them and to include Fe-enriched food in their dietary (Yip et al. 1998).

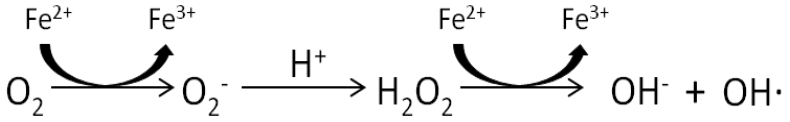
When the normal diet alone cannot restore deficient Fe levels to normal within certain time frame, Fe supplementation is indicated. Supplemental Fe is available in both ferrous (e.g. ferrous fumarate, ferrous sulfate and ferrous gluconate) and ferric (e.g. ferric bisglycinate) form (Melamed et al. 2007). Fe is better absorbed in acidic conditions, thus Fe is recommended to supply with ascorbic acid such as orange juice. Meanwhile, any foods or medications that can reduce the Fe absorption should be avoided (e.g. tea tannins or phytates). Occasionally, parenteral therapy is indicated in case of oral therapy failure (Clark 2008).

Because rice is a major crop and primary food source for more than 50% of the world population (Lee et al. 2009b), the increase of bio-available Fe in rice grains would improve Fe nutrition for the people suffering from IDA (Goto et al. 1999; Briat et al. 2015). To achieve this goal, many approaches were adopted. Fe-storage protein ferritin genes from soybean or rice were over-expressed under the control of the glutelin promoter (*OsGluB1, OsGluA2*) in rice plants (Goto et al. 1999; Lucca et al. 2002; Vasconcelos et al. 2003; Qu et al. 2005; Paul et al. 2012). More than 1.5-fold increase of Fe concentration in rice grains (brown or polished), were obtained in transgenic lines compared to non-transgenic rice. Nicotianamine (NA) is a main Fe chelator and key component for Fe homeostasis in rice plant (Kobayashi and Nishizawa 2014). Through introducing and over-expressing the NA synthase (NAS) genes from sorghum (*HvNAS1*) in rice plants, the Fe content in polished seeds was elevated by about 2-fold in transgenic lines (Masuda et al. 2009). Lee et al. (2009b) isolated two activation-tagged mutant lines in which *OsNAS3* expression was enhanced by 35S enhancer elements and found that the seeds Fe concentration was increased about 3-fold. Similar findings were shown in three populations of rice in which constitutively over-expression of *OsNAS1*, *OsNAS2* and *OsNAS3* were generated (Johnson et al. 2011). Mugineic acid family phytosiderophores (MAs) play an important role in Fe uptake and transport. One barley MAs synthase gene (*IDS3*) was introduced into a *japonica* cultivar 'Tsukinohikari' and increased the Fe concentration in polished seeds about 1.4-fold (Masuda et al. 2008). Other Fe transport genes, such as *OsIRT1* and *OsYSL15* were over-expressed in rice plants leading to the increase of Fe concentration in brown seeds of 1.1- and 1.3-fold, respectively (Lee and An 2009; Lee et al. 2009a). *OsIRO2*, a basic helix-loop-helix (*bHLH*) transcription factor, which positively regulates the MAs secretion from roots, was over-expressed in rice plants and enhanced the Fe concentration in brown seeds about 3-fold (Ogo et al. 2011a). Other genes, such as rice vacuolar Fe transporter (*OsVIT1* and *OsVIT2*) were found to be linked with the transport of Fe from flag leaves to rice seeds (Zhang et al. 2012). Interestingly, the repression of these two genes increased Fe content in rice seeds (Zhang et al. 2012; Bashir et al. 2013). The approaches in the studies mentioned above only used single transgenic

methods. Through multi-transgenic approaches, even higher level of seed Fe concentration can be achieved. Wirth et al. (2009) simultaneously introduced two genes, NAS and ferritin, into rice plants and increased the Fe content in endosperm by more than 6-fold. Similar results were achieved by introducing *OsYSL2*, *SoyferH2* and *HvNAS1* into *japonica* cultivar 'Tsukinohikari' (Masuda et al. 2012) and Myanmar rice 'Paw San Yin' (Aung et al. 2013). The combination of different transgenic approaches showed even better performance than single transgenic approaches in seeds Fe biofortification.

### 1.8 Fe overload in human body

Even though it has functions in many vital processes, excess Fe is toxic due to its involvement in the formation of reactive oxygen species (ROS). $Fe^{2+}$ ions can catalyze the reduction of oxygen and hydrogen peroxide, so called 'Fenton reaction' shown below (Fontecave and Pierre 1993).




The generated hydroxyl radicals are extreme toxic and can initiate lipid peroxidation and DNA damage that links to the development of cancer in humans (Stohs and Bagchi 1995; Valko et al. 2004). Moreover, excess Fe deposition was also associated with several neurodegenerative diseases including common disorders such as Parkinson, Alzheimer disease and some rare disorders, e.g. aceruloplasminemia, Hallervorden-Spatz disease (neurodegeneration with brain iron accumulation), Friedreich ataxia, and neuroferritinopathy (Zecca et al. 2004). Additionally, Fe overload can worsen the susceptibility of infectious diseases (e.g. tuberculosis, malaria) and the response to infection and inflammation (Wessling-Resnick 2010).

The human body has highly conserved mechanisms to obtain Fe safely, thus avoiding Fe overload/toxicity (Fishbane et al. 2014). The regulation of Fe homeostasis that is controlled by hepcidin, which is a 25-amino-acid peptide produced by hepatocytes, starts with the intestinal Fe absorption and involves the macrophage Fe recycling and hepatocyte Fe mobilization (Hunter et al. 2002; Andrews and Schmidt 2007). Hepcidin binds the Fe exporter protein ferroportin, resulting in the tyrosine phosphorylation, ubiquitination, and subsequent degradation of ferroportin (Papanikolaou et al. 2005; De Domenico et al. 2007). Hepcidin deficiency leading to the over-expression of ferroportin that increases the dietary Fe absorption, results in Fe overload in hereditary hemochromatosis and ineffective

erythropoiesis (Ganz and Nemeth 2011). The mutations of ferroportin, transferring receptor, ferritin and hemeojuvelin genes are responsible for genetic Fe overload (Andrews and Schmidt 2007). Except for the genetic factors, intravenous injection of Fe in the therapy of hemodialysis and chronic kidney disease can also increase the possibility of Fe overload (Fishbane et al. 2014). The treatments of Fe overload include the application of Fe-chelators, e.g. desferrioxamine, N,N'-Bis(2-hydeoxybenzyl)ethyler N,N'-diacetic acid, α-ketohydroxypyridones and pyridoxal isonicotinoyl hydrazone (Chaston and Richardson 2003), and in severe cases, the phlebotomy therapy is recommended (Angelucci et al. 1997).

### 1.9 Fe deficiency in rice

In well aerated neutral and calcareous soils, Fe is present exclusively in the oxidized form $Fe^{3+}$ with low solubility (Briat and Lobréaux 1997). When Fe supply is limited, rice plants exhibit Fe deficiency symptoms manifested as chlorosis due to chlorophyll deficiency, which severely reduces yield production and quality (Kobayashi et al. 2007). To cope with Fe deficient conditions, rice plants utilize a Fe chelating strategy (Strategy II, Fig. 2) in the acquisition of Fe from soil. The key enzymes involved in the process are deoxymugineic acid (DMA) synthases (*OsNAS1*, *OsNAS2*, *OsNAAT1* and *OsDMAS1*), DMA efflux transporter (TOM1) and $Fe^{3+}$-DMA transporter (*OsYSL15*) (Kobayashi et al. 2014). Additionally, rice plant also employs $Fe^{2+}$ transporters (*OsIRT1*, *OsIRT2* and *OsNARMP1*) to mobilize the $Fe^{2+}$ ions generated through the reduction of $Fe^{3+}$ (Kobayashi and Nishizawa 2012). The translocation of Fe to xylem and phloem is performed by the mugineic acid (MA) family transporters nicotianamine (NA) efflux transporters and citrate efflux transporters (Kobayashi et al. 2014; Zheng et al. 2009). Two distinct classes of transcription factors regulate the expressions of Fe uptake and transport genes. Iron deficiency-responsive element-binding factor (IDEF1 and IDEF2) and Fe deficiency-inducible basic helix-loop-helix (bHLH) transcription factor (*OsIRO2*) are positive transcriptional regulators (Ogo et al. 2011b; Kobayashi et al. 2007; Ogo et al. 2008). Haemerythrin motif-containing really interesting new gene (RING) and zinc-finger protein gene (*OsHRZ1* and *OsHRZ2*), as well as another two transcription factors (*OsIRO3* and *OsbHLH133*) negatively regulate the expressions of Fe uptake and transport genes (Zheng et al. 2010; Kobayashi et al. 2013; Wang et al. 2013). Based on the understandings of Fe uptake and transport pathways, some studies reported the enhanced tolerance to Fe deficiency in rice employing transgenic approaches. Two nicotianamine amino-transferase (NAAT) genes from barley were transformed into rice generating Fe deficiency tolerant lines, which had about 4.1 times higher yield than non-transformant rice under Fe deficient conditions (Takahashi et al. 2001).

Ishimaru et al. (2007) reported that through the fusion of one yeast $Fe^{3+}$ chelate-reductase gene to the promoter region of *OsIRT1* and its introduction into rice, the transgenic lines showed higher $Fe^{3+}$ chelate-reductase activity and higher Fe uptake rate resulting in higher grain yield (7.9 times) than in non-transformed rice plants. Iron regulated transporter (IRT1) gene from peanut (*Arachis hypogaea* L.) was introduced into rice plants and high tolerance to low Fe availability in calcareous soil was achieved (Xiong et al. 2014).

## 1.10 Fe toxicity in rice

### *1.10.1 Fe toxicity occurrence and symptoms*

Under anaerobic conditions, such as the flooded soils in lowland rice, Fe is present in excess due to the low redox potential, which enables the reduction of $Fe^{3+}$ to its soluble form $Fe^{2+}$. Excess $Fe^{2+}$ ions are taken up and transported via the transpiration stream (Becker and Asch 2005). Free $Fe^{2+}$ ions react with hydrogen peroxide ($H_2O_2$), which are produced in several cell components including chloroplast and mitochondria, to generate hydroxide (OH-) and hydroxyl (OH·) (Becana et al. 1998). Hydroxyl radicals are extremely reactive and cannot be scavenged by plant antioxidants (Apel and Hirt 2004). The reactive radicals can irreversibly damage lipids, protein and nucleotides leading to cell death (Quinet et al. 2012). Under Fe toxic conditions, rice plants exhibit reduced photosynthesis owing to both stomatal and non-stomatal limitations (Pereira et al. 2013). The typical symptoms associated with Fe toxicity are ‘bronzing’ on rice leaves (Fig. 5A) (Ponnamperuma et al. 1955). Moreover, excess Fe in soil can damage the root system (Fig. 5B) thus affecting the uptake of other nutrients, such as phosphorus, zinc and copper (De Dorlodot et al. 2005). Fe toxicity can occur in various types of soils including acid sulfate, acid clayey, peat and poorly drained sandy soils (Becker and Asch 2005). In West Africa, Fe toxicity is one main constraint limiting rice production and the yield losses associated with Fe toxicity were estimated at 12-100% depending on rice cultivar, soil Fe concentration and occurring stages of rice growth (Audebert and Fofana 2009).


Figure 5. Rice leaves and roots affected by Fe toxicity. (A) Rice leaves showing different bronzing symptoms, healthy (top), moderate bronzing (middle) and high bronzing (bottom); (B) Rice roots grown in non-toxic (top), moderate toxic (middle) and severe toxic (bottom) soils.

### *1.10.2 General tolerance mechanisms to Fe toxicity*

In West Africa and Southeast Asia, e.g. Ivory Coast, Madagascar, Nigeria, Philippines and Vietnam, Fe toxicity is a common constraint to rice production (Audebert and Fofana 2009; Olaleye et al. 2009; Onaga et al. 2013). To cope with Fe toxicity in rice fields, certain field managements can be applied including drainage to avoid rapid decline of soil redox potential and application of fertilizers such as P, K,

Ca, Mg and Zn (Becker and Asch 2005; Audebert and Fofana 2009). However, the most effective and economical way is to breed tolerant cultivars.

The tolerance to Fe toxicity can be classified into two main aspects: root- and shoot-based mechanisms. The exclusion of $Fe^{2+}$ at root level is considered as the first defense line for excess Fe uptake. The re-oxidation and precipitation of $Fe^{2+}$ leads to the formation of a physical barrier – root plaque (Wu et al. 2012). The formation of root plaque is favored by root oxidizing power including radial oxygen loss (ROL) and enzymatic oxidation (Armstrong 1967). Root oxidizing power is mainly related to the molecular oxygen transported from shoots to roots through aerenchyma and then diffused through root tips and lateral roots (Chen et al. 1980). The tolerance in some cultivars that showed fewer leaf symptoms together with low shoot Fe concentrations, e.g. CK 4, Pokkali, can be linked to exclusion mechanisms (Audebert and Sahrawat 2000; Engel et al. 2012). The retention of Fe in roots was also proposed as another aspect of root-based tolerance (Becker and Asch 2005). Through either exclusion or retention in roots, less Fe will be transported from roots to shoots, thus preventing oxidative stress in leaves.

Shoot-based tolerance mechanisms involve Fe partitioning in different tissues, e.g. less photosynthetically active leaf sheaths and stems (Engel et al. 2012). Inside cells, apoplastic spaces and vacuoles are considered as the main compartment for excess metal ions storage in rice and *Arabidopsis* (Briat and Lobréaux 1997; Roschzttardtz et al. 2009; Moore et al. 2014). As a ubiquitous protein in living organisms, ferritin can store up to 4,000 Fe atoms in a safe and bio-available way. The storage of excess Fe in ferritin was associated with shoot-based mechanisms (Arosio et al. 2009; Silveira et al. 2009). Besides the partitioning of Fe in shoots at tissue or sub-cellular level, plants may also employ antioxidants or antioxidant enzymes for ROS scavenging. Since plants do not possess effective approaches to detoxify hydroxyl radicals (Apel and Hirt 2004), the elimination of hydrogen peroxide - the precursors of hydroxyl radicals, through catalases and peroxidases is essential (Halliwell 2006).

### *1.10.3 Genetic mapping of tolerant loci to Fe toxicity*

QTL mapping using bi-parental rice population to identify the tolerance to Fe toxicity have been conducted by many researchers (Wu et al. 1997; Wu et al. 1998; Wan et al. 2003a; Wan et al. 2003b; Dufey et al. 2009; Dufey et al. 2015). A number of QTLs were identified across all 12 rice chromosomes with various traits, such as leaf bronzing symptoms, relative shoot / root dry weight, shoot Fe concentration, antioxidants content and antioxidant enzymes activity (Dufey et al. 2015) indicating the complex genetic architecture of Fe toxicity tolerance. Surprisingly, no major loci for Fe toxicity tolerance in rice have been fine-mapped or cloned yet. Despite the

complexity of genetic architecture of Fe toxicity tolerance, some convergences of tolerant loci were detected among previously identified QTLs on several chromosomes. It indicates the common loci for Fe toxicity tolerance independent of different mapping populations and screening conditions (Dufey et al. 2015).

Traditional QTL mapping employs bi-parental populations representing relatively low genetic variability. It also has the limitation of a small number of genetic markers (several hundred) and few chromosomal recombination events thus resulting in low mapping resolution (Korte and Farlow 2013). Moreover, establishing populations for QTL mapping is usually laborious and cost-ineffective. To overcome these limitations, another mapping approach, genome-wide association study was applied to mitigate the shortcomings of bi-parental QTL mapping (Zhao et al. 2011; Wang et al. 2014). Even though both approaches investigate the QTL effects underlying certain phenotypes, to avoid the confusion, mapping with bi-parental populations is shortened as QTL mapping while genome-wide association mapping as GWAS in the following text. GWAS searches for genotype-phenotype correlations in unrelated individuals with diverse genetic variability (Myles et al. 2009). Unlike QTL mapping using linkage map, GWAS is based on the principle of linkage disequilibrium (LD), which is the nonrandom association between alleles at different loci (Visscher et al. 2012). Generally, loci that locate closely exhibit stronger LD than loci that are farther apart. LD is caused by evolutionary processes, such as mutation, drift and domestication while it is disrupted by recombination (Hartl and Clark 1997). The chromosomal extent of LD is crucial as it determines the number of genetic markers for association mapping and limits how finely loci may be detected (Nordborg et al. 2002). GWAS pioneered in the genetic analysis of human diseases (Burton et al. 2007; Scott et al. 2007). Nowadays, GWAS using diverse genotypes has evolved as a new and powerful tool to exploit the functional variation in loci associated with diverse traits in *Arabidopsis*, maize and wheat (Atwell et al. 2010; Kump et al. 2011; Tian et al. 2011; Sukumaran et al. 2015). There are more than 127,000 rice accessions that are held in the International Rice Genebank maintained by the International Rice Research Institute (IRRI, http://irri.org/our-work/research/genetic-diversity/international-rice-genebank). In addition, large-scale genotyping with single nucleotide polymorphism (SNP) array or next-generation sequencing provides dense genetic map for GWAS (McNally et al. 2009; Zhao et al. 2011). Conclusively, diverse genetic variations, comprehensive and high density of markers make GWAS suitable for rice genetic research regarding morphological, developmental and agronomic traits and tolerance to biotic/abiotic stress (Huang et al. 2010; Zhao et al. 2011; Wang et al. 2014; Ueda et al. 2014). For Fe toxicity study in rice, GWAS might be also a useful tool to investigate possible genetic variations linked to the tolerant loci that are lacking in other bi-parental populations. However, GWAS suffers from the limitation

that marker-trait associations may arise from the confounding population structure leading to false positives (Zhao et al. 2007). A number of statistical approaches were employed to reduce the confounding by population structure. One simple method is to rescale the *P*-values without changing the ranking of makers (Devlin and Roeder 1999). Other methods are to employ the model-based Bayesian clustering algorithm, STRUCTURE (Pritchard et al. 2000), or the principle component analysis, PCA (Price et al. 2006) to investigate population structure and relative kinship. An improved mixed-model approach was also introduced by (Yu et al. 2006), using a random effect to estimate the fraction of the phenotypic variation that can be explained by genome-wide relatedness. It should be noted that any method effectively reducing the confounding from population structure (Type I error) will also reduce the power of detecting the true positives (Type II error) strongly correlated with population structure (Zhao et al. 2007). This might be problematic for the genetic analysis of certain quantitative loci, in which variation is strongly correlated with population structure. A useful alternative is to employ the combination of QTL mapping with GWAS that reduces the occurrence of false negatives (Brachi et al. 2010; Famoso et al. 2011). Additionally, the combined strategies also have the potential benefit to narrow down the QTL intervals identified in QTL mapping. Thus, combining GWAS with bi-parental QTL mapping is necessary and promising to discover the complex nature of Fe toxicity tolerance in rice to contribute for the breeding of more adaptive cultivars.

## 1.11 Research objectives

In this thesis, I will focus on three main objectives regarding the tolerance mechanism to Fe toxicity in rice.

(I) Due to the contradictory tolerance rankings even in the same genotypes that were reported in other studies using different screening conditions, this study will focus on an acute Fe stress (1000 ppm, 5 days) simulating the conditions in inland valley receiving Fe-rich runoff water from adjacent slopes. The first part of this thesis (Chapter II) is to investigate the genetic factors of Fe toxicity tolerance using two distinct bi-parental populations for QTL mapping. Through co-localization analysis with previously reported QTLs, identification of chromosomal regions carrying common tolerant loci in various Fe toxic conditions will be plausible. The physiological mechanisms underlying root-based tolerance also need to be unraveled.

(II) Different genotypes that are contrasting in the tolerance despite similar shoot Fe concentrations will be selected and subjected to in-depth physiological analysis to test the three hypotheses (Chapter III):

(1) Fe uptake, partitioning and storage in different tissues or sub-cellular components conferring the tolerance.
(2) The tolerance is related to the biosynthesis of antioxidants that can scavenge the ROS generated by excess Fe.
(3) The tolerance is associated with antioxidant enzymes that detoxify ROS.

(III) To overcome the limitations of traditional bi-parental population mapping, genome-wide association study (GWAS) using a panel of 329 rice accessions genotyped by 44,100 single nucleotide polymorphism (SNP) markers will be conducted to unravel the genetic basis of Fe toxicity tolerance (Chapter IV). Candidate tolerant loci for leaf bronzing symptoms will be identified and employed for further investigations regarding the co-localization with previous QTLs. Physiological and biochemical analyses will be conducted to elucidate the possible tolerance mechanisms underlying these loci.

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# Chapter II. Genetic and physiological analysis of tolerance to acute iron toxicity in rice

**Wu L-B**, Shhadi MY, Gregorio G, Matthus E, Becker M, Frei M (2014) Genetic and physiological analysis of tolerance to acute iron toxicity in rice. Rice 7 (1): 1-12.

## 2.1 Abstract

Iron toxicity occurs in lowland rice production due to excess ferrous iron ($Fe^{2+}$) formation in reduced soils. To improve genotye selection and accelerate rice breeding for iron toxicity tolerance, we determined quantitative trait loci (QTL) by screening two different bi-parental mapping populations under iron pulse stresses (1,000 ppm $Fe^{2+}$ for 5 days) in hydroponic solution, followed by experiments with selected lines to determine whether QTLs were associated with iron exclusion (*i.e*. root based mechanisms), or iron inclusion (*i.e*. shoot-based mechanisms).

In an IR29/Pokkali $F_8$ recombinant inbred population, 7 QTLs were detected for leaf bronzing score on chromosome 1, 2, 4, 7 and 12, respectively, individually explaining 9.2-18.7% of the phenotypic variation. Two tolerant recombinant inbred lines carrying putative QTLs were selected for further experiments. Based on Fe uptake into the shoot, the dominant tolerance mechanism of the tolerant line FL510 was determined to be exclusion with its root architecture being conducive to air transport and thus the ability to oxidize $Fe^{2+}$ in rhizosphere. In line FL483, the iron tolerance was related mainly to shoot-based mechanisms (tolerant inclusion mechanism). In a Nipponbare/Kasalath/Nipponbare backcross inbred population, 3 QTLs were mapped on chromosomes 1, 3 and 8, respectively. These QTLs explained 11.6-18.6% of the total phenotypic variation. The effect of QTLs on chromosome 1 and 3 were confirmed by using chromosome segment substitution lines (SL), carrying Kasalath introgressions in the genetic background on Nipponbare. The Fe uptake in shoots of substitution lines suggests that the effect of the QTL on chromosome 1 was associated with shoot tolerance while the QTL on chromosome 3 was associated with iron exclusion.

Tolerance of certain genotypes were classified into shoot- and root- based mechanisms. Comparing our findings with previously reported QTLs for iron toxicity tolerance, we identified co-localization for some QTL in both pluse and chronic stresses, especially on chromosome 1.

Keywords:

Iron toxicity, *Oryza sativa* L., quantitative trait locus, reactive oxygen species, tolerance mechanism.

## 2.2 Introduction

Iron is an essential element in plants that is involved in many physiological processes, but that can also be toxic when provided in excess. In well-aerated soils, Fe is present as ferric hydroxides with low plant availability (Conte and Walker 2011). However, in anaerobic soils and at low pH, Fe is reduced to its soluble form $Fe^{2+}$ and can be taken up excessively by plants. In plant tissues, $Fe^{2+}$ participates in Fenton reactions, catalyzing the generation of hydroxyl radicals (·OH) and other reactive oxygen species (ROS) (Briat and Lebrun 1999; Thongbai and Goodman 2000). These radicals cause irreversible damage to membrane lipids, proteins and nucleic acids (Becana et al. 1998). Eventually they oxidize chlorophyll and subsequently reduce leaf photosynthesis (Pereira et al. 2013), thereby leading to yield reductions. The typical symptoms associated with iron toxicity are leaf discoloration (bronzing) and reddish spots (Ponnamperuma et al. 1955). Yield losses associated with iron toxicity commonly range from 15% to 30%. However, in the case of severe toxicity at younger stage, complete crop failure can occur (Audebert and Sahrawat 2000).

In the field, several types of Fe toxic conditions occur, differing by landscape and soil type attributes, the Fe concentrations in the solutions, and the physiological stage at which the stress occurs. Three types of iron toxic conditions were proposed (Becker and Asch 2005). (i) Due to high content of $Fe^{2+}$ in acid sulfate soils, toxicity symptoms on plants can be observed during the whole growth period. Yield losses range from 40% to 100%. (ii) In acid clay soils, high iron concentrations typically occur at around one month or more after transplanting. Leaf bronzing symptoms appear mainly during the late vegetative growth stage while genotypes are transplanted in the dry season with high vapor pressure deficits. (iii) In inland valleys, interflow water containing large amounts of $Fe^{2+}$ can be flushed at the onset of the rainy season from adjacent slopes formed on highly weathered soils into the poorly drained valley bottoms with often sandy soils with low cation exchange capacity and low available P. Symptoms can be observed at the early development stages. Yield losses can range from 30-70%, but when severe toxicity occurs at the seedling stage, total crop failure can occur. While various soil, water and nutrient management strategies have been suggested to counteract negative effects of excess iron in solution, the most promising approach is to use tolerant genotypes.

To adapt rice plants to these varying iron toxic conditions, three types of tolerance mechanisms have been proposed. Type I refers to exclusion of $Fe^{2+}$ at the root level. Root oxidizing power due to oxygen release or enzymatic oxidation is responsible for the oxidization and precipitation of $Fe^{2+}$ on the root surface, thus avoiding excess $Fe^{2+}$ from uptake into rice shoots (Ando et al. 1983; Green and Etherington 1977). Type II refers to the inclusion but subsequent avoidance of $Fe^{2+}$ *via* internal

distribution and storage in a less reactive form. Thus, ferritin is a promising candidate protein as it can accommodate up to 4,000 Fe atoms in a safe and bio-available form (Briat et al. 2010). Type III refers to inclusion and tolerance to ROS formed in the Fenton reactions. Anti-oxidants such as ascorbic acid, and reduced glutathione can scavenge ROS (Fang et al. 2001; Gallie 2013), and antioxidant enzymes, such as superoxide dismutase, peroxidase and catalase reportedly protect plants from ROS damage (Bode et al. 1995; Fang and Kao 2000; Fang et al. 2001).

A number of screening and mapping experiments for tolerance to iron toxicity in rice have reported somewhat contradictory tolerance rankings. For example, the *japonica* variety, Azucena was screened in 250 ppm $Fe^{2+}$ for 4 weeks in hydroponics and classified as tolerant (Wu et al. 1997; Dufey et al. 2009). However, it showed high susceptibility under a pulse stress of 1,500 ppm $Fe^{2+}$ (Engel et al. 2012). Another two varieties, WITA 1 and Matkandu from Africa Rice Center and Malaysia, respectively, were moderately tolerant in iron-toxic soils in Korhogo, Ivory Coast, but responded sensitively to iron stress in Kilissi, Guinea (Audebert and Fofana 2009). Yet another *indica* genotype, Pokkali was screened to be sensitive on an acid sulfate soil with chronic toxicity in the Philippines (Gregorio et al. 2002), but it showed marked tolerance when treated with an iron pulse stress of 1,500 ppm $Fe^{2+}$ in hydroponics (Engel et al. 2012). These examples illustrate contradictory performances of the same genotypes in different screening studies. Due to the diversity of conditions under which iron toxicity occurs and the different stress types and intensities, different crop adaptive strategies are required. To overcome the limitations posed by contradictory tolerance rankings, a deeper understanding of the physiological mechanisms of adaptation to different iron toxic conditions and the genetic factors behind those mechanisms is required.

The mapping of QTL is an effective way to dissect genetic factors underlying phenotypic traits such as iron stress tolerance. Several previous studies have reported QTLs for tolerance in diverse iron toxic conditions (Dufey et al. 2009; Dufey et al. 2012; Fukuda et al. 2012; Shimizu 2009; Wan et al. 2003; Wu et al. 1997; Wu et al. 1998) but the physiological tolerance mechanisms behind these QTLs have not been specified and remain still unclear. Our aim was therefore to dissect genetic factors associated with tolerance to a specific type of iron toxicity by mapping of QTL, and to classify the physiological tolerance mechanism underlying these QTLs. We focused on intensive pulse stresses at the vegetative stage, as they typically occur during rainfall events in inland valleys (Audebert and Sahrawat 2000). We first screened the parents of two mapping populations segregating in tolerance to iron pulse stress, subsequently conducted two QTL mapping experiments with these populations, and finally investigated the physiological mechanisms underlying tolerance using selected lines from the mapping populations.

## 2.3 Material and Methods

### 2.3.1 Plant material

Two pairs of parents from mapping populations were screened initially for tolerance to an iron pulse stress at the vegetative growth stage: IR29/Pokkali, and Nipponbare/Kasalath. Based on contrasting tolerance response patterns, these populations were selected to identify tolerance QTL for iron pulse stresses. An $F_8$ recombinant inbred (RIL) population consisting of 121 lines was derived from a cross of two *indica* varieties IR29 (intolerant) and Pokkali (tolerant) (Gregorio 1997). Pokkali was characterized as tolerant to intensive pulse stresses during the vegetative growth stage due to its iron exclusion capacity (Engel et al. 2012). Another population consisted of 98 BC1F5 lines derived from a backcross of Nipponbare/Kasalath//Nipponbare by the single-seed descent method (Taguchi-Shiobara et al. 1997). Nipponbare was characterized as a moderately tolerant *japonica* variety (Engel et al. 2012), whereas Kasalath was a highly sensitive *Aus* variety. Further chromosome segment substitution lines (SL) carrying small Kasalath inserts in a Nipponbare genetic background were provided by the Rice Genome Resource Center of NIAS, Japan.

### 2.3.2 Hydroponic culture and screening

Experiments were conducted in a glasshouse with the day/night temperature set as 30/25 °C, and natural light with supplementary lighting to ensure a minimum photosynthetically active radiation (PAR) of 400 µmol $m^{-2}$ $sec^{-1}$. Rice seeds were soaked in demineralized water and germinated at 30ºC in the dark for 72 hours. Subsequently, germinating seeds were floated in 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$ solution in light for another 5 days. Homogenous seedlings were selected and transplanted into 40 L tanks filled with half-strength nutrient solution (Yoshida et al. 1976). In all experiments, at least four replicate plants per genotype were used, except for the QTL mapping of the IR29/Pokkali population, where the number of available containers could only accommodate 3 replicates per line. After 10 days, nutrient solutions were changed to full-strength with the following composition: 40 mg $L^{-1}$ N (as $NH_4NO_3$), 10 mg $L^{-1}$ P (as $NaH_2PO_4{\cdot}2H_2O$), 40 mg $L^{-1}$ K (as $K_2SO_4$), 40 mg $L^{-1}$ Ca (as $CaCl_2$), 40 mg $L^{-1}$ Mg (as $MgSO_4 \cdot 7H_2O$), 0.5 mg $L^{-1}$ Mn (as $MnCl_2 \cdot 4H_2O$), 0.05 mg $L^{-1}$ Mo (as $(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_2O$), 0.2 mg $L^{-1}$ B (as $H_3BO_3$), 0.01 mg $L^{-1}$ Zn (as $ZnSO_4 \cdot 7H_2O$), 0.01 mg $L^{-1}$ Cu (as $CuSO_4 \cdot 5H_2O$), 2 mg $L^{-1}$ Fe (as $FeCl_3 \cdot 6H_2O$ with 14.9mg $L^{-1}$ citric acid monohydrate). During the whole growing period, the pH value was adjusted to 5.5 every other day and solutions were completely exchanged after 10 days. The roots of plants grown in the same container were separated using PVC tubes fixed underneath a perforated covering plate.

After 5 weeks-of growth, half of the rice plants were exposed to a pulse stress of 1,000 ppm $Fe^{2+}$ (as $FeSO_4 \cdot 7H_2O$) for 5 days. Maintaining a low redox potential (Eh) in solution prevents $Fe^{2+}$ from being re-oxidized and reportedly accelerates toxicity symptom expression (Wang et al. 2008). Consequently, $N_2$ gas was percolated into the culture solutions for 15 minutes every 2 hours to remove dissolved oxygen to keep the solution at low redox potential. On the second and fifth day after adding the $Fe^{2+}$, leaf bronzing scores were measured on the three youngest fully expanded leaves of the main tiller. Leaf bronzing scores indicate the severity of iron toxicity and range from 0 (healthy leaf without symptom) to 10 (dead leaf) (Wissuwa et al. 2006). Plant materials were harvested for further investigation. Shoot dry weight and root dry weight were measured. The reduction of shoot and root growth were calculated as: Relative Shoot Dry Weight = (shoot dry weight in treatment)/(shoot dry weight in control)×100; Relative Root Dry Weight =(root dry weight in treatment)/(root dry weight in control) ×100.

### 2.3.3 QTL mapping and statistical analysis

For the IR29/Pokkali population, a physical map consisted of 173 SNP and 83 SSR markers covering all 12 rice chromosomes based on the physical locations. QTL analysis was performed by single marker regression analysis (Kearsey and Hyne 1994) with Qgene 4.3 (Joehanes and Nelson 2008). A -log p(F) ≥ 3.0 was taken as the threshold for the detection of putative QTL. For Nipponbare/Kasalath population, the genetic linkage map was composed of 245 RFLP markers (Lin et al. 1998). QTLs were analyzed by the composite interval mapping method (Zeng 1994) with Qgene 4.3. LOD value ≥ 2.5 was used as the threshold of for the declaration of putative QTL. Statistical software SPSS was applied for analysis of variance (ANOVA, IBM SPSS Statistics 21). Pair-wise genotypic differences were determined by post-hoc comparison using the LSD-Test and Tukey adjustment was used for multiple comparison of means if appropriate.

### 2.3.4 Physiological analysis of contrasting lines

Plant shoots of contrasting lines were oven-dried at 60ºC until the weight was constant and ground to a fine powder. Fe concentrations in shoots were determined after digesting 250 mg of dry samples with 4 ml 65% $HNO_3$ at 180ºC for 8 hours followed by dilution to 25 ml and filteration. Standard and sample solutions were measured using atomic absorption spectroscopy (AAS, Perkin-ELMER 1100B, Überlingen, Germany).

The redox indicator methylene blue was applied to detect the root oxidizing power (Kotula 2009). Solution containing 0.75% agar was boiled and then cooled down to 60ºC with continuous percolation of $N_2$ gas to completely remove the dissolved oxygen. Methylene blue was added into agar solution at a concentration of 2 mg $L^{-1}$.

The blue mixture containing the oxidized dye was furtherly cooled down to 35ºC. Then, 0.75 g $L^{-1}$ sodium dithionite ($Na_2S_2O_4$) was added to reduce methylene blue and the solution turned to colorless. The roots of 4 representative plants of each line were carefully placed in 500 ml Erlenmeyer flasks. Gaseous $N_2$ was applied to remove air from the flasks. Colorless solution containing reduced methylene blue was poured into the flasks to submerge the whole root system. The open surface of flasks was immediately covered with a plastic wrap to avoid air diffusion. Flasks were wrapped with aluminum foil to keep the roots in the dark. The plants were placed in a greenhouse at 30 ºC for 4 hours. Photographs were taken at every hour to record the color changes in rhizosphere due to the root oxdizing power.

To observe the aerenchyma formation in root and shoot, 4 plants of each line were selected. Primary roots were detached at 2 mm distance from shoot base and sliced vertically using a razor blade. The main tiller of each line was also sliced at the shoot base. The slices were observed using a light microscope (Leica DFC425, Heerbrugg, Germany) and photographs were taken to show the aerenchyma formation and measure the diameter of primary root and pith cavity in shoot. Root system of contrasting lines were scanned (EPSON, EU-88, Seiko Epson Corp. Japan) and lateral fine roots within 1 cm on primary roots were counted at 10 different sites of each plant. Root total length of each plant was measured by XLRhizo 2012b (Regent Instruments Inc. Canada).

## 2.4 Results

### 2.4.1 The screening of parental lines

The parental lines of two mapping populations were screened with a pulse stress of 1,000 ppm $Fe^{2+}$ in hydroponics. Genotypes IR29 and Pokkali showed significant difference ($P$ <0.01) in leaf bronzing score after 2- and 5-days-treatments (Figure 1a, b). The relative root and shoot dry weights of Pokkali were significantly higher than those of IR29 (Figure 1c, d). Also the bronzing scores after 2 and 5 days of stress exposure and the root biomass of Nipponbare were significantly lower than of those of Kasalath (Figure 1a, b), but no significant difference was found in shoot biomass (Figure 1d). Pokkali showed markedly higher tolerance than IR29 in terms of symptom score and relative shoot and root growth. Nipponbare was more tolerant than Kasalath in terms of symptom score and root growth. Therefore, the populations derived from these parents were considered suitable for QTL analysis.

### 2.4.2 QTL analysis in IR29/Pokkali population

In the IR29/Pokkali population, significant QTLs were only detected for the trait leaf bronzing score after 5 days of stress exposure. A total of 7 QTLs were mapped on chromosome 1, 2, 4, 7, and 12, respectively (Table 1). Standard nomenclature for QTLs was adopted (McCouch et al. 1997). On chromosome 1, two putative QTLs were detected: *q*FETOX-1-1 was located at the position of 12.9 Mb explaining 10.6% of the phenotypic variation. Another QTL, *q*FETOX-1-2 was highly associated with two linked markers at the position of 36.8 - 38.2 Mb. It explained 12.7-16% of the phenotypic variation. On chromosome 2, *q*FETOX-2 was found at 31.2 Mb, which explained 10.3% of the phenotypic variation. *q*FETOX-4-1 covering four linked markers was mapped at 7.4-12.0 Mb on chromosome 4. At the position of 20 Mb on chromosome 4, *q*FETOX-4-2 which explained 9.9% of the phenotypic variation was detected. Another two QTLs, *q*FETOX-7 and *q*FETOX-12 were mapped at 3.7 Mb on chromosome 7 and 27.6 Mb on chromosome 12, respectively. Except for *q*FETOX-1-2, the tolerance alleles of all QTLs were provided by Pokkali.

Table 1. QTLs associated with leaf bronzing scores after 5 days of $Fe^{2+}$ stress exposure in IR29/Pokkali population by single marker regression.

| QTL[a] | Chromosome | Associated marker[b] | Position[c] (Mb) | -log *p*(F)[d] | Additive effect[e] | $R^2$ (%)[f] | Tolerance allele[g] |
|---|---|---|---|---|---|---|---|
| *q*FETOX-1-1 | 1 | id1008684 | 12.9 | 3.57 | 0.82 | 10.6 | Pokkali |
| *q*FETOX-1-2 | 1 | id1021920 | 36.8 | 4.23 | -0.72 | 12.7 | IR29 |
| | 1 | id1023158 | 38.2 | 5.25 | -0.81 | 16.0 | |
| *q*FETOX-2 | 2 | id2013434 | 31.2 | 3.47 | 0.94 | 10.3 | Pokkali |
| *q*FETOX-4-1 | 4 | id4002852 | 7.4 | 6.12 | 0.85 | 18.7 | Pokkali |
| | 4 | id4002913 | 8.1 | 6.14 | 0.86 | 18.7 | |
| | 4 | id4003259 | 10.0 | 5.95 | 0.84 | 18.1 | |
| | 4 | id4003727 | 12.0 | 3.15 | 0.82 | 9.2 | |
| *q*FETOX-4-2 | 4 | id4005867 | 20.0 | 3.37 | 0.69 | 9.9 | Pokkali |
| *q*FETOX-7 | 7 | id7000519 | 3.7 | 3.87 | 1.11 | 11.7 | Pokkali |
| *q*FETOX-12 | 12 | id12010050 | 27.6 | 3.56 | 0.96 | 10.6 | Pokkali |

[a] Closely linked markers are assumed as the same QTL;

[b] Marker associated with QTL;

[c] Physical position of markers on chromosomes;

[d] F-statistical analysis indicates association between markers and trait;

[e] Positive/negative values indicate IR29/Pokkali can increase trait values;

[f] Proportion of phenotypic variance explained;

[g] Tolerance allele provided by parental line.

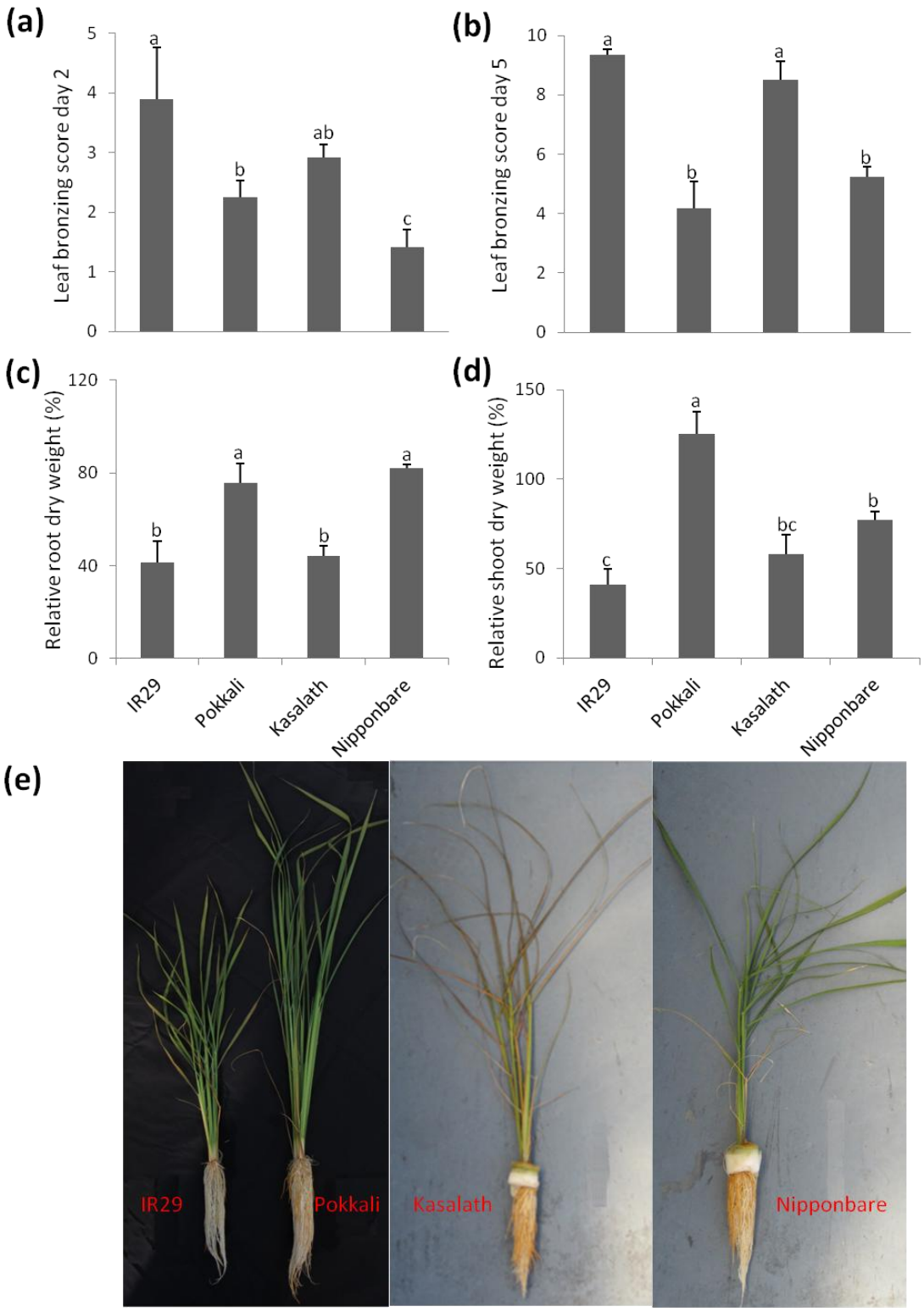


Figure 1. Parents of two mapping populations were screened after a pulse stress of 1,000 ppm $Fe^{2+}$ for 5 days. (a) Leaf bronzing score after 2-days-treatment, (b) leaf bronzing score after 5-days-treatment, (c) relative root dry weight after 5-days-treatment and (d) relative shoot dry weight after 5-days-treatment were determined. Bars represent standard errors of the mean (n=6). Different letters above data points indicate significant differences between genotypes by LSD-test ($P$ <0.05).

### 2.4.3 Fe uptake analysis in contrasting lines in IR29/Pokkali population

Based on the screening, FL510 and FL483, which showed significantly lower leaf bronzing scores (Figure 2a) than both parents, were chosen for Fe uptake analysis. FL510 carried the tolerance alleles of all QTLs detected in IR29/Pokklai population, while FL483 only carried tolerance alleles at *q*FETOX-1-1 and *q*FETOX-1-2 (Table 1). Compared to IR29, the shoot Fe concentration was significantly lower in Pokkali and FL510, while FL483 did not differ significantly from any genotype (Figure 2b). Lower Fe concentrations in Pokkali despite higher absolute Fe uptake (Figure 2d) may have partly occurred due to higher biomass (Figure 2c) leading to a 'dilution' effect. However, FL510 had even lower Fe concentration than Pokkali (Figure 2b), despite a significantly lower biomass than Pokkali (Figure 2c), suggesting that dilution was not the dominant factor leading to low Fe concentrations in FL510. FL483 did not differ significantly from IR29 in shoot Fe concentration, dry weight, or shoot Fe uptake, suggesting that it was tolerant due to a shoot-based mechanism.

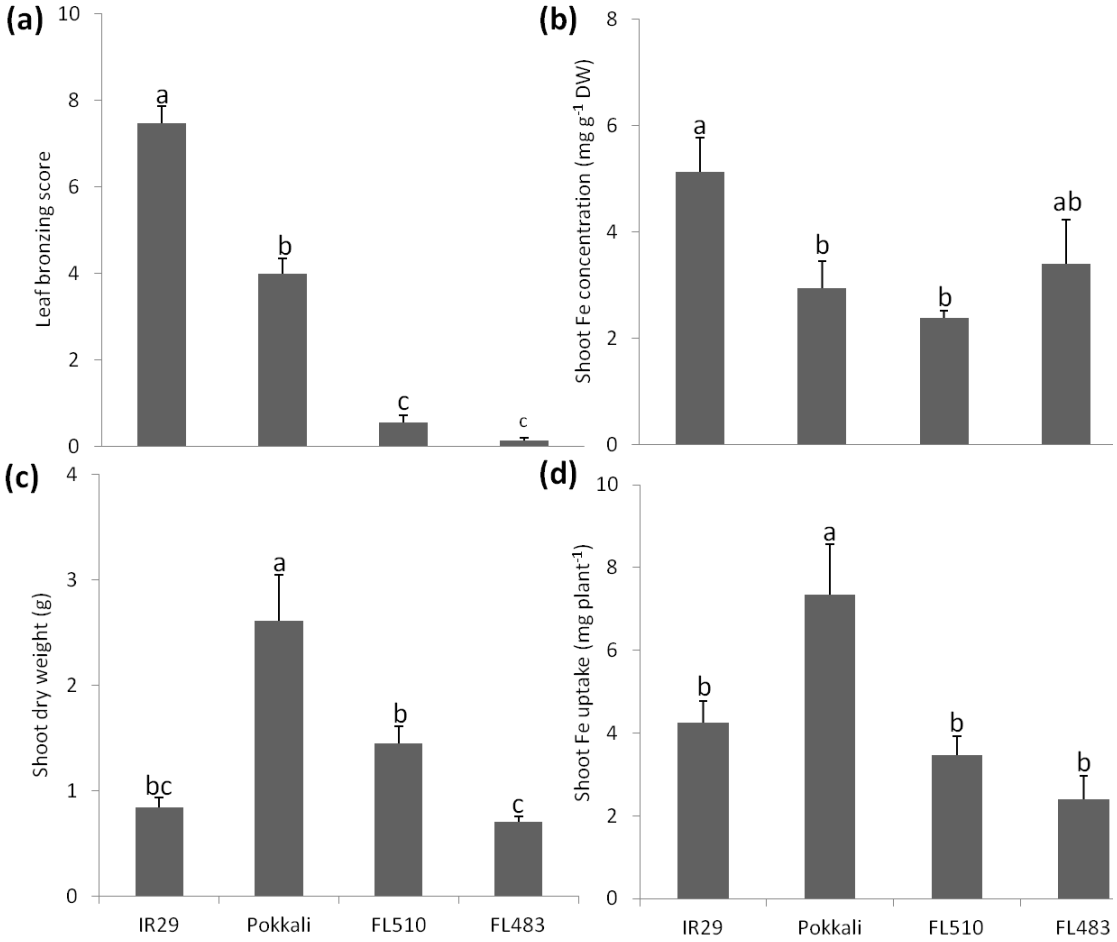


Figure 2. Phenotypic traits of contrasting lines in IR29/Pokkali population under 1,000 ppm $Fe^{2+}$ stress for 5 days were determined. (a) leaf bronzing score; (b) Fe concentration in shoot; (c) shoot dry weight; (d) total Fe uptake in shoot. Vertical bars represent standard errors of means (n=4). Different letters above the data points indicate significant differences between genotypes by LSD-test ($P$ <0.05).

### 2.4.4 Root oxidizing power and microscopy test

To investigate the physiological basis of low shoot Fe concentration in FL510, the oxidizing power of roots as a main factor in excluding Fe in the rhizosphere was investigated. Oxidation of the rhizosphere as indicated by a color change of the Methylene-blue indicator proceeded at a faster pace in Pokkali than in IR29, while FL510 was intermediate (Figure 3). The color change was first observed around the root tips and lateral fine roots, suggesting that these sites are important for root oxygen release. To investigate the mechanisms associated with differences in root oxidizing power among IR29, Pokkali and FL510, plant shoots and roots were tested for architectural traits (Figure 4a, b). The results showed that at the same growth stage, Pokkali and FL510 had aerenchyma with a larger diameter in the *pith* cavity than IR29 (Figure 4c). The degree of aerenchyma differentiation in primary roots among the lines was not obviously different (Figure 4b). However, the difference of primary root diameters was significant ($P$ <0.05) between contrasting lines (Figure 4d). The density of lateral fine roots was measured by counting their number within 1 cm of the primary roots. As shown in Figure 4e, the lateral fine roots of Pokkali and FL510 were denser than of IR29. Even though the trend of total root length was consistent with density of lateral fine roots, the difference among these lines was not significant (Figure 4f).

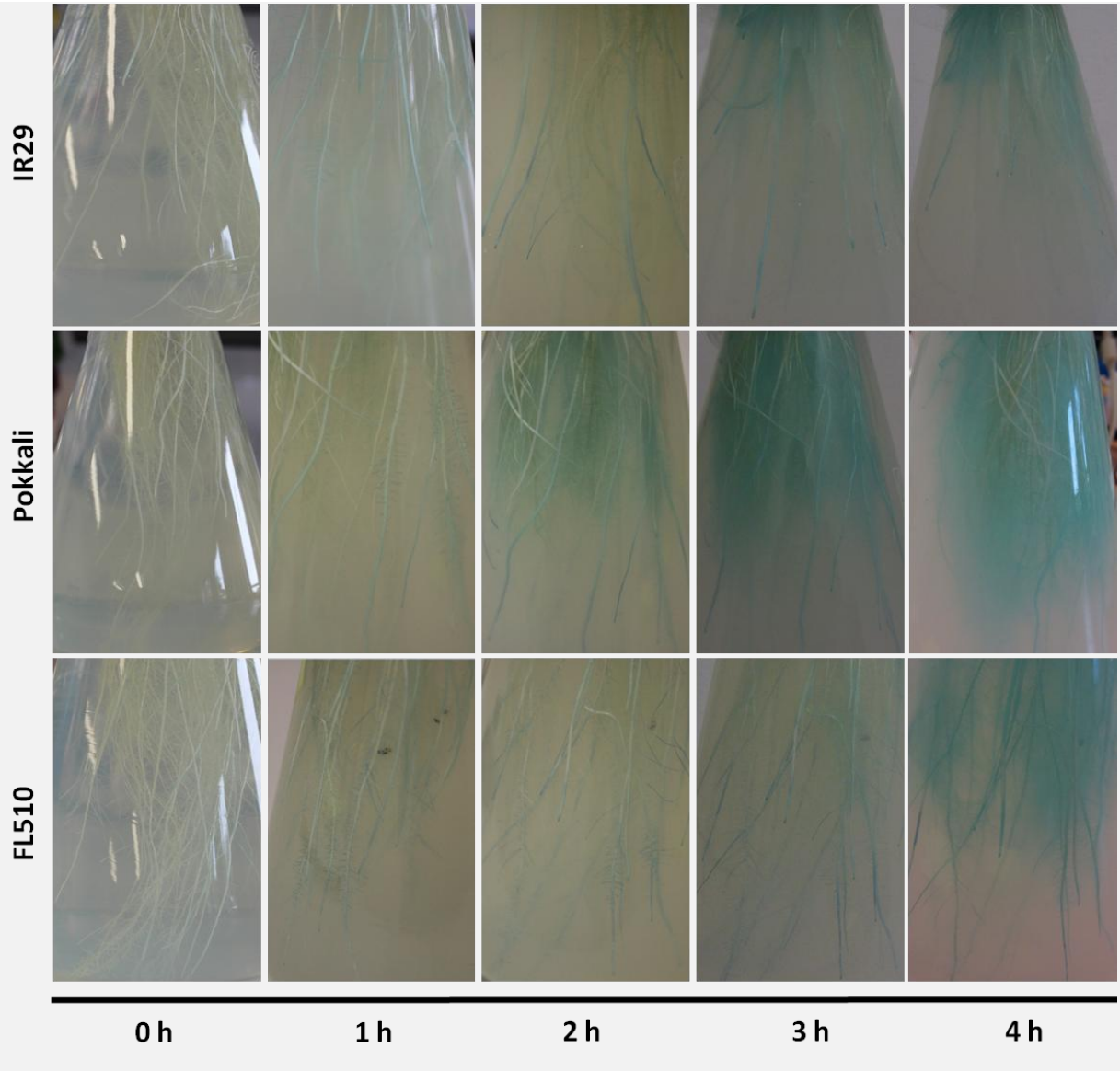


Figure 3. Time course of root oxidizing power of contrasting lines in IR29/Pokkali population were indicated by color change in Methylene-blue agar solution. Representative photos of 4 replicates per genotype are shown. Horizontal axis represents the time of duration (0-4 hours). Blue color indicates the site of oxygen release from roots.

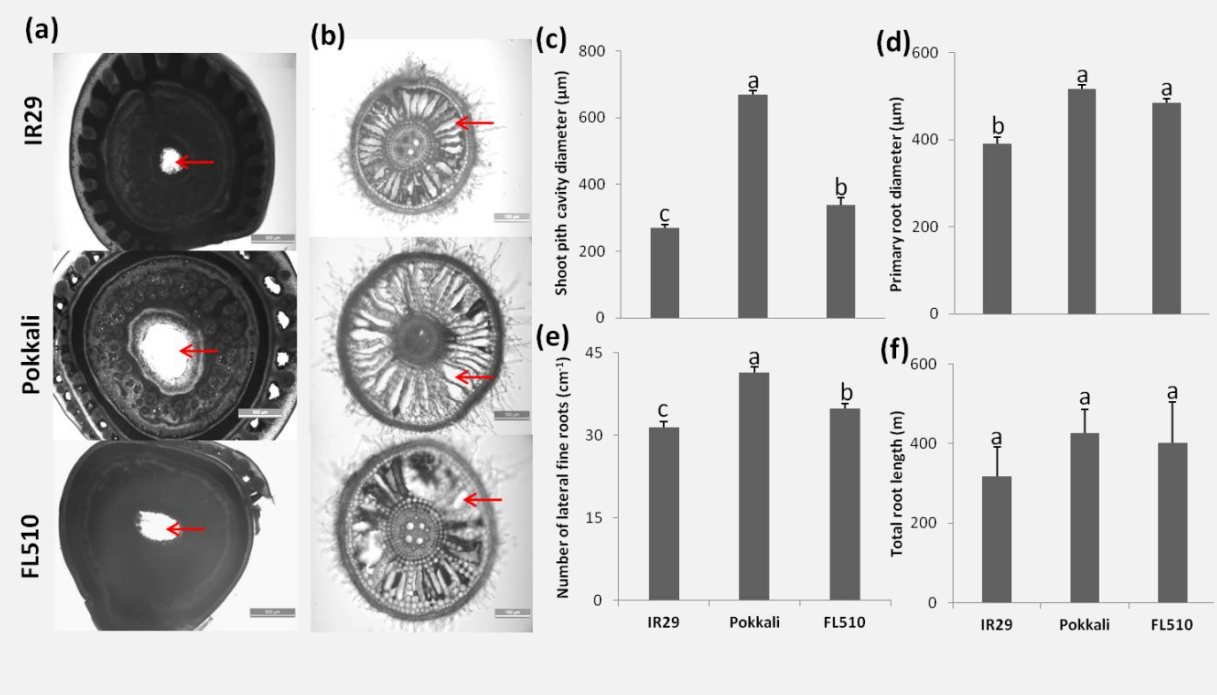


Figure 4. Architectural traits related to rhizosphere aeration of contrasting lines in IR29/Pokkali population. Representative images of the pith cavity in shoots (a) and primary roots (b) in the three lines were documented after 5days of Fe stress. Red arrows indicate the aerenchyma. The diameter of pith cavity (c) and primary roots (d) were determined and vertical bars indicate standard errors of means (n=4). The numbers of lateral fine roots within 1 cm distance from primary roots were counted (e), and vertical bars represent standard errors of means (n=20). The total length of roots (f) was determined by root scanning; vertical bars indicate standard errors of means of n=3 (IR29 and FL510) or n=4 (Pokkali). Different letters indicate significant differences between genotypes by LSD-test ($P$ <0.05).

### 2.4.5 QTL analysis in Nipponbare/Kasalath population

In the Nipponbare/Kasalath population (Table 2), one putative QTL *q*FETOX-1-3 was mapped in the marker interval of C742-C86 by using the trait of leaf bronzing score after 2-days-treatment on chromosome 1. It explained 18.6% of the phenotypic variation. On chromosome 3, *q*FETOX-3 was mapped between C136 and R250 for both leaf bronzing score after 2- and 5-days-treatment. On chromosome 8, *q*FETOX-8 with an LOD value of 3.96 was detected, and it explained 17% of the phenotypic variation. For the QTLs on chromosome 1 and 8, tolerance alleles were provided by Nipponbare, whereas Kasalath contributed the tolerance allele to the QTL on chromosome 3.

Table 2. QTLs associated with leaf bronzing scores in Nipponbare/Kasalath population by composite interval mapping.

| QTL | Trait[a] | Chr[b] | Marker interval[c] | Support interval (cM)[d] | LOD value | $R^2$ (%)[e] | Additive effect[f] | Tolerance allele[g] | CSSLs[h] |
|---|---|---|---|---|---|---|---|---|---|
| *q*FETOX-1-3 | LBS2 | 1 | C742-C86 | 16.7-26.9 | 4.38 | 18. 6 | -0.53 | NB | SL54 |
| *q*FETOX-3 | LBS2 | 3 | C136-R250 | 23.7-37.7 | 2.63 | 11. 6 | 0.44 | Kas | SL15 |
| | LBS5 | 3 | C136-R250 | 23.7-37.7 | 3.71 | 16.0 | 0.52 | Kas | SL15 |
| *q*FETOX-8 | LBS5 | 8 | R727-C166 | 44.0-70.7 | 3.96 | 17.0 | -0.51 | NB | SL38 |

[a] LBS2: leaf bronzing score after 2 days of treatment, LBS5: leaf bronzing score after 5 days of treatment;

[b] Chromosomes number on which QTLs were detected;

[c] QTL was located between the markers;

[d] The position of marker associated with QTL on linkage map;

[e] Proportion of phenotypic variance explained;

[f] Positive/negative values indicate Nipponbare/Kasalath can increase trait values;

[g] Tolerance allele provided by parental line, NB Nipponbare, Kas Kasalath;

[h] Chromosome segment substitution lines carrying Kasalath genetic inserts related to specific QTLs.

### 2.4.6 Confirmation of QTL using chromosome segment substitution lines

To confirm the QTLs detected in Nipponbare/Kasalath population, chromosome segment substitution lines (SL) carrying Kasalath inserts in Nipponbare genetic background were used. SL54 carried the intolerance allele at the position of *q*FETOX-1-3, SL15 carried the tolerance allele at the location of *q*FETOX-3 and SL38 carried the intolerance allele at the position of *q*FETOX-8 (Supplementary Figure S1). SL54 showed significantly higher ($P$ <0.01) leaf bronzing scores after 2 and 5 days than Nipponbare (Figure 5a, b) in accordance with the expected QTL effect (Table2). For *q*FETOX-3, SL15 showed significantly lower bronzing scores ($P$ <0.01) than Nipponbare in accordance with the expected effect (Table 2). However, no significant differences in relative root and shoot biomass were found between two SLs and Nipponbare (Figure 5c, d). Overall, the effect of the two QTLs, *q*FETOX-1-3 and *q*FETOX-3 were confirmed. For *q*FETOX-8, the associated substitution line SL38 showed similar leaf bronzing score after 2-days-treatment as Nipponbare (Figure 5a). However, bronzing scores (5 days) of SL38 was lower than in Nipponbare which was contrary to expected QTL effect (Figure 5b). Also for the root biomass, SL38 showed significantly higher values than Nipponbare, while no significant differences were observed for shoot biomass (Figure 5c). Due to inconclusive data, the effect of *q*FETOX-8 was not confirmed.

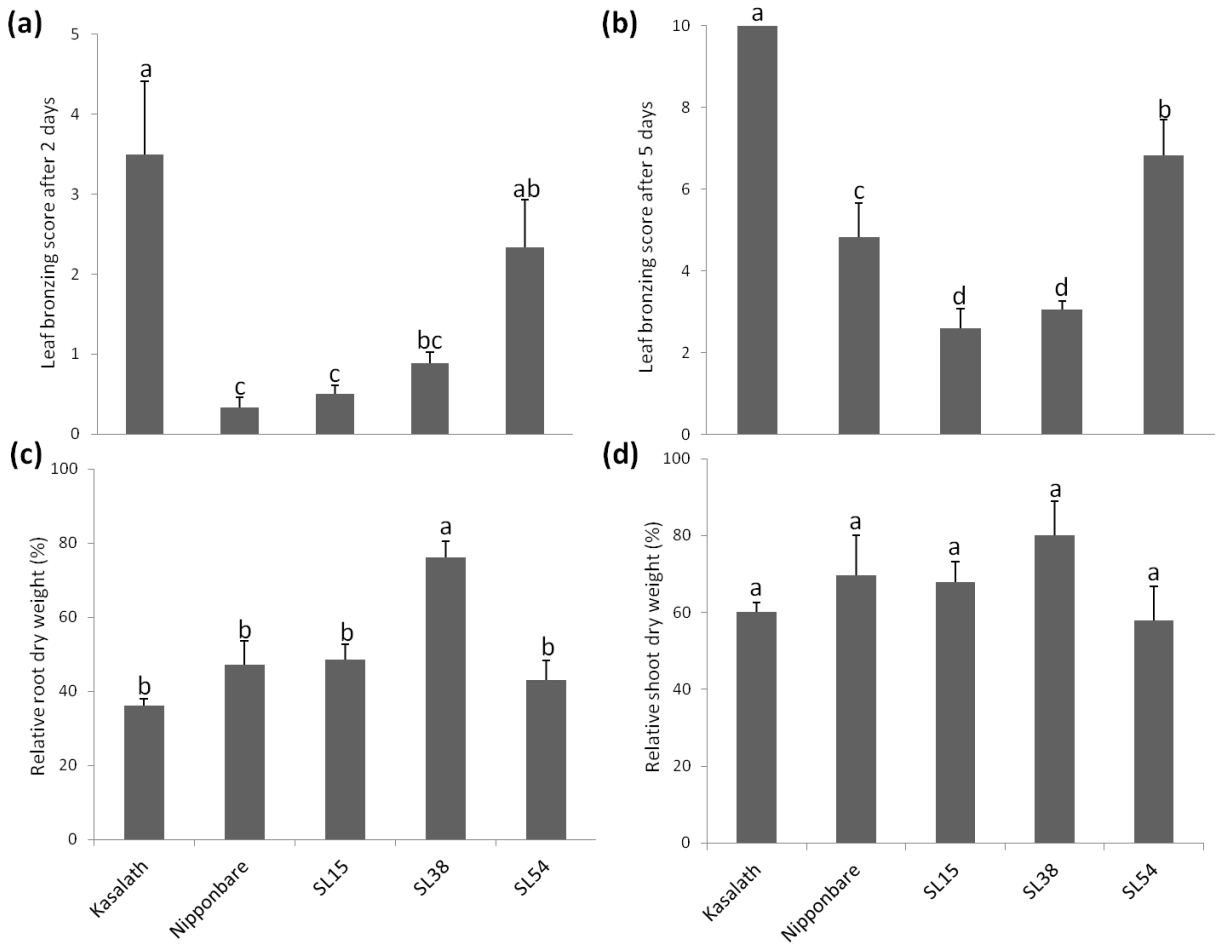


Figure 5. Phenotypic traits of chromosome segment substitution lines SL15 and SL54 and two parents tested in 1,000 ppm $Fe^{2+}$ stress for 5 days. (a) Leaf bronzing score after 2-days-treatment, (b) leaf bronzing score after 5-days-treatment, (c) relative root dry weight and (d) relative shoot dry weight were determined. Vertical bars indicate standard errors of means (n=4). Different letters indicate significant differences between genotypes by LSD-test (*P* <0.05).

### 2.4.7 Analysis of shoot Fe uptake in SLs in Nipponbare / Kasalath population

Iron concentration was analyzed in those SLs carrying confirmed QTL along with their parents to gain some insight into the associated mechanism. Kasalath was extremely sensitive to iron stress despite significantly lower iron concentration than Nipponbare (Figure 6a). The low concentration may be a result of high biomass (Figure 6b), leading to a 'dilution effect'. Similarly, both SLs had significantly lower iron concentration than Nipponbare, although they did not differ significantly from Nipponbare in terms of biomass (Figure 6b). These SLs showed contrasting tolerance although they both had similar iron concentrations. These data collectively suggested that the *q*FETOX-1-3 (SL54) was associated with shoot sensitivity, leading

to higher stress level despite lower iron concentration, while *q*FETOX-3 (SL15) conferred tolerance via iron exclusion.

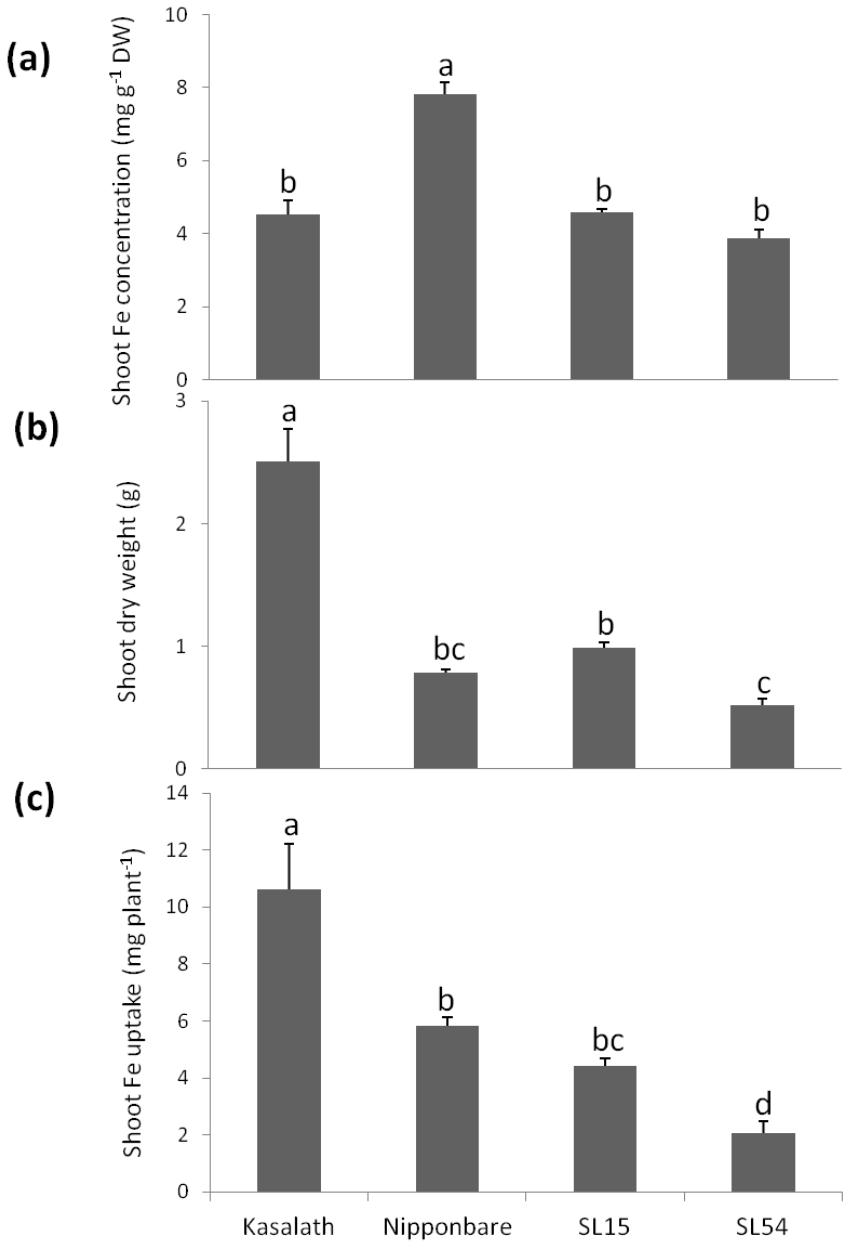


Figure 6. Shoot Fe concentration, dry weight, and Fe uptake and dry weight in SL15 and SL54 and their two parents after treatment with 1,000 ppm $Fe^{2+}$ stress for 5 days. (a) Shoot Fe concentration, (b) shoot dry weight, and (c) total Fe uptake were determined. Vertical bars indicate standard errors of means (n=4). Different letters indicate significant differences between genotypes by LSD-test ($P$ <0.05).

## 2.5 Discussion

Leaf bronzing symptoms are often used as a phenotypic trait in studies on abiotic stresses. They can quickly be scored for a large number of plants and are associated with many abiotic stresses in rice, *e.g* Zn deficiency and ozone stress (Frei et al. 2010; Höller et al. 2013). Under iron-toxic conditions, leaf bronzing was strongly correlated with yield loss. It was estimated that each visual symptom score increment is associated with a yield loss of approximately 400 kg $ha^{-1}$ (Audebert and Fofana 2009). Thus, we considered leaf-bronzing score as a relevant trait for the screening of tolerance to iron toxic conditions.

Contradictory rankings of tolerance were observed in many studies due to different screening conditions and different development stages at which plants were tested. Our study was targeting the intensive pulse stress at the early vegetative stage that typically occurs during rainfall events in a tropical inland valley landscape. We simulated this iron pulse in nutrient solutions that was maintained at low redox potential by gaseous $N_2$ percolation (Engel et al. 2012). Genetic analyses of tolerance to iron toxicity have been reported in several previous studies, although most of them used different screening conditions, and they focused on chronic rather than pulse stresses. Putative QTLs detected in our study were compared with previous reports based on the physical positions of associated markers in the Nipponbare genome (International Rice Genome Sequencing Project). Based on leaf bronzing and other physiological traits, QTLs associated with tolerance to iron toxicity were mapped on whole genome except for chromosome 5 and 6 (Figure 7). On chromosome 1, *q*FETOX-1-2 in IR29/Pokkali population and *q*FETOX-1-3 in Nipponbare/Kasalath population co-localized with the QTLs detected by Dufey et al. (2009, 2012) and Wu et al. (1998). Another QTL in the IR29/Pokkali population, *q*FETOX-2 was mapped close to a QTL reported by Wan et al. (2005). For other QTLs in our study, no co-localization was found with previous reports, except for *q*FETOX-7 which was mapped close to one QTL for physiological trait by Wu et al. (1998). The fact that there is multitude of rather small effect QTLs underlines the concept of multiple tolerance mechanisms involved in different types of iron toxicity, for example chronic versus pulse stress. This view is also supported by a transcriptomic study in which gene regulation under short term iron stress was substantially different from chronic iron stress in rice (Quinet et al. 2012).

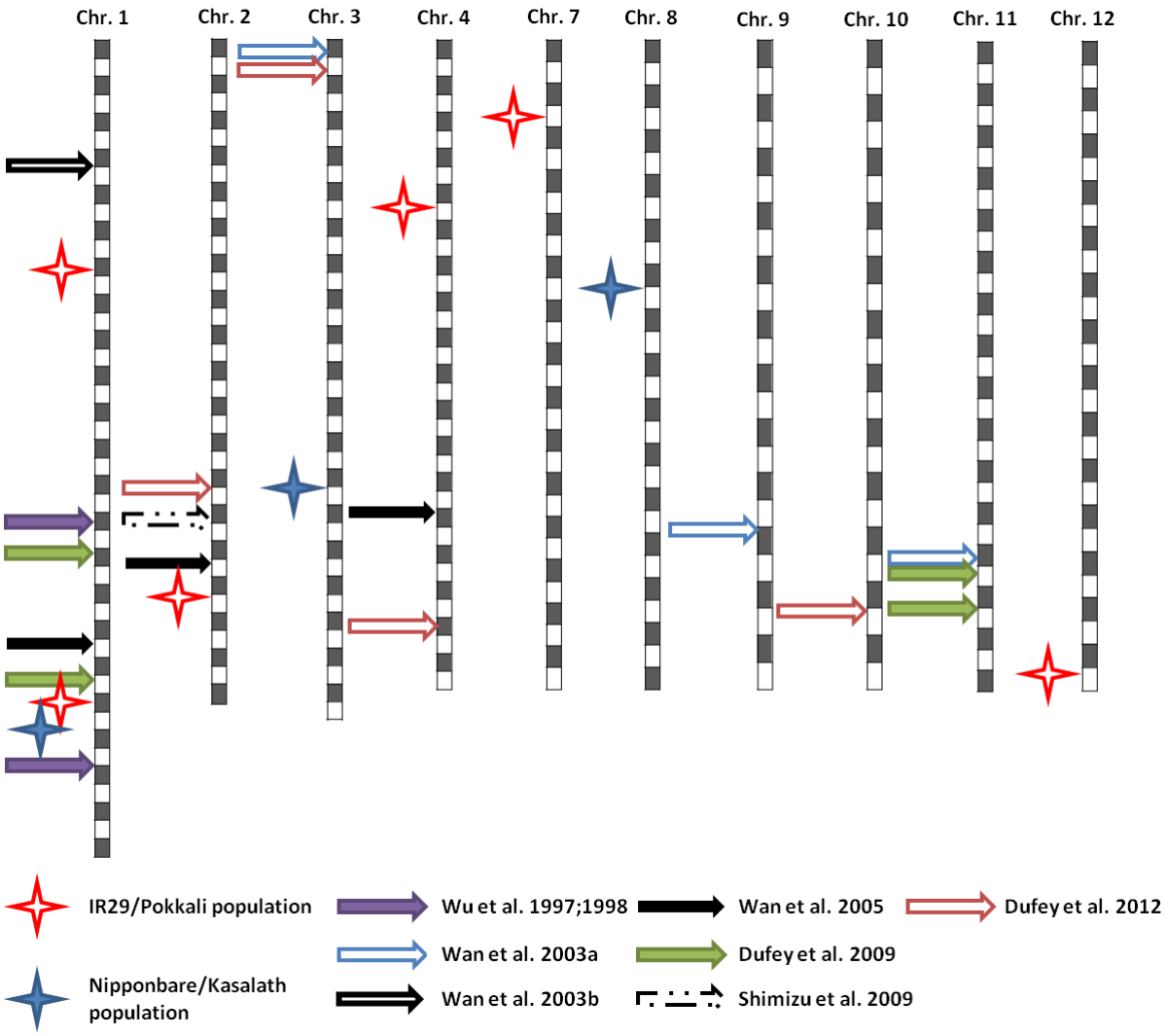


Figure 7. Co-localization analysis of QTL reported in this study with previously reported QTLs for leaf bronzing under Fe toxic conditions in rice. QTL were located on chromosomes based on the physical positions of flanking markers. One quadrate (in grey or white) represents 1 Mb. Stars represent the QTLs mapped in this study and arrows represent the QTLs from other previous reports.

To shed some light on mechanism involved in tolerance to iron pulse stress, we investigated iron uptake and root morphological properties associated with iron oxidation. Oxidation of $Fe^{2+}$ at root surface typically leads to the formation of iron plaque that has also been proposed to act as a barrier or buffer to reduce the uptake of toxic elements (*e.g* Al, Cd, As) into plant tissues (Liu et al. 2004; Liu et al. 2010; Chen et al. 2006). Root oxidizing power as a direct cause of $Fe^{2+}$ oxidation was composed of two distinct processes: root oxygen release and enzymatic oxidation (Ando et al. 1983). The aerenchyma provides a low resistance pathway for internal oxygen movement within the roots (Colmer 2002). Our results suggest that larger diameter of pith cavity in shoot that favors the oxygen transport from shoot to root,

along with larger primary root diameter, which could increase the absolute volume of aerenchyma, together with higher density of lateral fine roots was effective in increasing the root oxidation power and thus increasing iron exclusion ability in Pokkali and FL510. Similarly, a QTL (*q*FETOX-3) associated with iron exclusion was identified in the Nipponbare/Kasalath population, which was represented SL15. The same SL15 was previously shown to be quite sensitive to ozone stress (Frei et al. 2008), indicating that it was not tolerant to oxidative stress in the shoot, but that tolerance in this line was clearly associated with iron exclusion.

Our study also revealed a several QTL apparently associated with shoot based mechanisms, which may be composed of two components: iron compartmentation and ROS detoxification. Higher amount of iron in low-molecular-mass fractions (<3 kDa) was found in the leaf extracts of sensitive genotype suggesting iron stored in low-molecular-mass fractions as a possible mechanism for shoot based tolerance (Stein et al. 2009). Ferritin as iron storage protein may play an important role in iron compartmentation as the higher accumulation of ferritin mRNA and protein was observed in tolerant genotype (da Silveira et al. 2009). Antioxidant enzyme activities and antioxidants are important for ROS detoxicification. It is reported that ascorbate peroxidase, glutathione reductase and peroxidase activities were increased in rice leaf by excess iron (Fang et al. 2001; Fang and Kao 2000; Stein et al. 2009). External application of mannitol and reduced glutathione could scavenge the damage from iron toxicity (Fang et al. 2001). However, to our knowledge, there is no direct evidence of constitutively high content of antioxidants or antioxidant enzymes activities leading to higher tolerance to iron toxicity. Our study suggests that shoot based mechanisms may be effective in protecting plants from iron pulse stress. In particular, FL483 carrying the favorable allele of the *q*FETOX-1-2 showed markedly high tolerance with similar shoot Fe concentration with intolerant parent IR29. In Nipponbare/Kasalath population, SL54 carried the intolerant allele of *q*FETOX-1-3 which was located near *q*FETOX-1-2 showed certain shoot sensitivity, suggesting that this segment on chromosome 1 was related to shoot-based tolerance. Further investigations are warranted to determine whether these loci are related to iron compartmentation or ROS detoxification.

## 2.6 Conclusion

We identified a number of QTLs related to tolerance of iron pulse stresses in the vegetative growth stage of rice. Some of these QTL co-localize with previously reported QTL that were mapped under more chronic iron stress, suggesting that they were associated with ‘universal’ defense mechanisms. However, the majority of QTLs had rather small effects and was distributed throughout the genome, confirming the complexity of the genetics behind adaptation to varying iron toxic conditions. Further, QTLs were associated with either exclusion or inclusion mechanisms of iron tolerance. We suggest that iron exclusion via oxidation at the root surface is an important adaptive trait under iron pulse stress. The trait appears to be favored by root architecture and can be genetically dissected within the IR29/Pokkali mapping population. Pyramiding this trait with further shoot based adaptive traits may be effective in the breeding for iron toxicity tolerance.

## Acknowledgements

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# Chapter III. Shoot tolerance mechanisms to iron toxicity in rice (*Oryza sativa* L.)

**Wu L-B**, Ueda Y, Lai S-K, Frei M (2016) Shoot Tolerance Mechanisms to Iron Toxicity in Rice (*Oryza sativa* L.). Plant Cell Environ.

## 3.1 Abstract

Iron toxicity frequently affects lowland rice and leads to oxidative stress via the Fenton reaction. Tolerance mechanisms were investigated in contrasting genotypes: the intolerant IR29 and the tolerant recombinant inbred line FL483. Seedlings were exposed to 1,000 ppm ferrous iron and the regulation of genes involved in three hypothetical tolerance mechanisms were investigated (I) Iron uptake, partitioning and storage. The iron concentration and speciation in different plant tissues did not differ significantly between genotypes. Sub-cellular iron partitioning genes such as vacuolar iron transporters or ferritin showed no genotypic differences. (II) Antioxidant biosynthesis. Only one gene involved in carotenoid biosynthesis showed genotypic differences, but carotenoids are unlikely to scavenge the reactive oxygen species (ROS) involved in Fe toxicity, i.e. $H_2O_2$ and hydroxyl radicals. (III) Enzymatic activities for ROS scavenging and antioxidants turnover. In shoots, glutathione-S-transferase and ascorbate oxidase genes showed genotypic differences, and consistently, the tolerant FL483 had lower dehydroascorbate reductase and higher ascorbate oxidase activity, suggesting that high rates ascorbate reduction confer sensitivity. This hypothesis was confirmed by application of exogenous reduced ascorbate or L-galactono-1,4-lactone, which increased lipid peroxidation under iron toxic conditions. Our results demonstrate *in planta* pro-oxidant activity of reduced ascorbate in the presence of iron.

Key words: rice, iron toxicity, Fenton reaction, microarray, antioxidant, ascorbic acid, pro-oxidant.

## 3.2 Introduction

Iron (Fe) is an essential microelement that is involved in various important processes in plant cells. Through the redox status change between the ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) form, Fe functions as an electron donor or acceptor, which is crucial in the processes of respiration and photosynthesis (Kobayashi and Nishizawa 2012; Zhai et al. 2014). Moreover, Fe serves as a co-factor of many enzymes, e.g. nitrate reductase (nitrogen fixation) or ribonucleotide reductase (DNA synthesis) (Briat and Lobréaux 1997).

In well aerated, neutral and calcareous soils, Fe is present exclusively in the oxidized form $Fe^{3+}$, which has low solubility. To overcome the limited availability, higher plants have evolved two distinct Fe acquisition strategies (Römheld and Marschner 1986). Most non-graminaceous plants use Strategy I, which involves the acidification of the rhizosphere through the release of protons from the roots, the reduction of $Fe^{3+}$ to $Fe^{2+}$, and the transport of $Fe^{2+}$ ions across the root plasma membrane through iron regulated transporter (*IRT*). Graminaceous plants have developed Strategy II, i.e. Fe acquisition through the uptake of $Fe^{3+}$-phytosiderophore complexes. Although being considered as a Strategy II plant, rice (*Oryza sativa* L.) possesses $Fe^{2+}$ transporter genes (*OsIRT1*, *OsIRT2*) that can directly transport $Fe^{2+}$ from the soil (Bughio et al. 2002; Ishimaru et al. 2006). For sub-cellular Fe distribution, several transporter genes were identified, such as the vacuolar iron transporter (*OsVIT*) (Zhang et al. 2012), mitochondrial iron transporter (*OsMIT*) (Bashir et al. 2011) in rice and the permease in chloroplasts1 (PIC1) in *Arabidopsis* (Duy et al. 2007).

In the flooded soils of typical lowland rice fields, Fe availability is not limited because the redox potential is sufficiently low to reduce $Fe^{3+}$ to its soluble form $Fe^{2+}$. Despite its importance as a plant nutrient, Fe can be toxic in excess. Fe toxicity is one of the most commonly observed abiotic stresses affecting millions of hectares of rice fields, especially in West Africa and Asia (Becker and Asch 2005; Audebert and Fofana 2009). Rice yield losses due to Fe toxicity were reported to range from 12% to 100%, depending on the cultivated varieties and the stress intensities (Sahrawat 2004). In plant cells, many reactions use oxygen molecules as electron acceptors generating superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$). These reactive oxygen species (ROS) contribute to the production extremely reactive hydroxyl radicals ($OH^\bullet$) through the so-called Fenton reaction with the involvement of catalytic $Fe^{2+}$, which occurs excessively under Fe toxic conditions (Becana et al. 1998). The generated $Fe^{3+}$ can be reduced back by other reducing reagents with sufficiently low redox potential such as ascorbate (Halliwell 1996). ROS, especially $OH^\bullet$, can oxidize lipids, proteins and DNA and thus cause necrosis. In rice, Fe toxicity leads to the visible symptoms

termed as leaf bronzing, which occur along with reductions of shoot and root growth (Ponnamperuma et al. 1955; Wu et al. 2014).

Different mechanisms were proposed to confer tolerance to Fe toxicity (Becker and Asch 2005). In brief, root-based tolerance mechanisms include the formation of a physical barrier (root plaque) through the precipitation of oxidized $Fe^{3+}$ thus preventing further excessive Fe uptake. Root oxygen diffusion could play an important role in this tolerance mechanism and was suggested to be favored by root aerenchyma and lateral fine root formation, as well as enzymatic $Fe^{2+}$ oxidation (Green and Etherington 1977; Becker and Asch 2005; Wu et al. 2014). Another aspect of root-based tolerance is to retain Fe in specific 'dumping sites' in roots thus forming metabolically inactive Fe (Becker and Asch 2005).

Shoot-based tolerance mechanisms include the retention of Fe in less photosynthetically active tissues, i.e. leaf sheath or stem (Engel et al. 2012a). Inside the cells, excess Fe can be stored in vacuoles for avoiding stress by sub-cellular Fe partitioning (Moore et al. 2014). Besides the vacuole, the ferritin protein occurring in plastids can store up to 4,000 Fe atoms and was shown to be involved in the tolerance to Fe toxicity in rice (da Silveira et al. 2009; Briat et al. 2010). Another previously proposed shoot-based tolerance mechanism is the scavenging of ROS by antioxidants such as ascorbate, glutathione, phenolics, or antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) (Fang et al. 2001; Majerus et al. 2007).

Genetic aspects of Fe toxicity tolerance were investigated in various studies reporting quantitative trait loci (QTL), which were summarized by Wu et al. (2014) and Dufey et al. (2015). Several QTLs were co-localized in the interval of 36.8-41 Mb on chromosome 1, a region which was linked with shoot-based tolerance (Wu et al. 2014). A recombinant inbred line FL483 carrying the QTLs *qFETOX-1-1* and *qFETOX-1-2* in this chromosomal region showed less leaf bronzing symptoms despite similar shoot Fe concentration compared to its sensitive parental line IR29 (Wu et al. 2014). Genome-wide association study (GWAS) was also conducted by Matthus et al. (2015) employing a diverse panel of 329 rice accessions. Significant markers associated with leaf bronzing symptoms were detected on chromosomes 1 and 5. The loci associated with tolerance to Fe toxicity on chromosome 1 were localized in the same region, where several QTLs had been previously detected in different studies (Wu et al. 2014; Dufey et al. 2015). Transcriptomic analyses can shed further light on the genetic regulation of Fe toxicity in rice. In a previous microarray study, Quinet et al. (2012) compared short- and long-term transcriptional responses by exposing one cultivar to either 3-day or 3-week Fe stress. They showed that more genes were regulated under short-term than long-term Fe stress,

especially in root tissue. However, because only one genotype was employed, their study did not address genotypic differences in transcriptional regulation to differentiate between tolerant and sensitive responses.

Our study was specifically designed to investigate the mechanisms of shoot-based tolerance to Fe toxicity through transcriptional and physiological analyses of a contrasting pair of genotypes from a QTL mapping population. The tolerant genotype FL483 carried positive alleles at two QTLs, one of them being localized in the region associated with shoot tolerance on chromosome 1 (Wu et al. 2014). Specifically, we hypothesize that the following mechanisms could be associated with shoot-based tolerance in the FL483: (I) Fe uptake, partitioning and speciation in different cellular organelles or tissues, which are differing in photosynthetic activity; (II) Biosynthesis of antioxidants for ROS scavenging; and (III) enzymatic activities for ROS scavenging and antioxidants turnover to maintain 'healthy' redox homeostasis. Expanding upon these hypotheses, we also investigated the possibility of a pro-oxidant role of ascorbic acid (AsA) *in planta*.

## 3.3 Materials and methods

### *3.3.1 Plant material*

Two rice (*Oryza sativa* L.) genotypes were used, i.e. the high yielding variety IR29 (sensitive *indica*) and the recombinant inbred line FL483 (tolerant) derived from a cross between IR29 and the *indica* landrace Pokkali (Gregorio 1997). FL483 had previously been shown to possess shoot tolerance due to the presence of tolerant alleles at two QTLs, *qFETOX-1-1* and *q*FETOX-1-2 on chromosome 1 (Wu et al. 2014). Seeds were originally obtained from the International Rice Research Institute (IRRI), Los Baños, Philippines.

### *3.3.2 Plant culture in hydroponics*

Experiments were conducted in a climate controlled glasshouse at the Institute of Crop Science and Resource Conservation, University of Bonn (Bonn, Germany). Natural light was supplemented with artificial lighting to ensure a minimum photosynthetically active radiation of 400 µmol $m^{-2}$ $s^{-1}$. The day/night temperature was set to 28/22 °C. Rice seeds were soaked in de-mineralized water and germinated for 3 days in the dark at 30 °C. Subsequently, germinated seeds were floated in solutions containing 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$ in the light for another 7 days. Homogenous seedlings were selected and transplanted into the 60-liter tanks filled with half-strength Yoshida solution (Yoshida et al. 1976). After 10 days, half-strength nutrient solutions were replaced with full-strength solutions, which had the following composition: 2.86 mM N (as $NH_4NO_3$), 0.32 mM P (as $NaH_2PO_4 \cdot 2H_2O$), 1.02 mM K (as $K_2SO_4$), 1 mM Ca (as $CaCl_2$), 1.65 mM Mg (as $MgSO_4 \cdot 7H_2O$), 9.1 µM Mn (as $MnCl_2 \cdot 4H_2O$), 0.52 µM Mo (as $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$), 18.5 µM B (as $H_3BO_3$), 0.15 µM Zn (as $ZnSO_4 \cdot 7H_2O$), 0.15 µM Cu (as $CuSO_4 \cdot 5H_2O$), 35.8 µM Fe (as $FeCl_3 \cdot 6H_2O$ with 70.9 µM citric acid monohydrate). The pH value was adjusted to 5.5 every other day and nutrient solutions were completely renewed every 10 days. The root systems of single plants grown in the same container were separated using PVC tubes fixed underneath a perforated covering plate.

### *3.3.3 Microarray experiment*

Plants were pre-grown for five weeks of growth, and then ten plants of each genotype were exposed to 1,000 ppm $Fe^{2+}$ (as $FeSO_4 \cdot 7H_2O$) in three replicated containers, while the same amount of plants were grown in the control conditions. Nutrient solutions were automatically percolated with $N_2$ gas for 15 min every two hours to maintain low redox potential for preventing the $Fe^{2+}$ oxidation. Stress symptoms on three fully-expanded leaves of the main tiller were scored from 0 (healthy leaf) to 10 (dead leaf) according to Wu et al. (2014). On the fourth day after starting the treatment, three plants of each genotype per container were pooled. Shoots and

roots were separated and flash-frozen in liquid N for further RNA extraction and biochemical analyses. Other plants were harvested and dried for metal elements analysis.

*3.3.4 Hypothesis testing experiments*

Further experiments were conducted to test the effects of exogenous L-ascorbic acid (AsA) and its biosynthetic precursor, L-galactono-1,4-lactone (L-GalL) (Wheeler et al. 1998) on Fe toxicity tolerance. Different treatments were applied including (1) control, (2) 1,000 ppm $Fe^{2+}$, (3) exogenous AsA/L-GalL application as foliar spray, and (4) 1,000 ppm $Fe^{2+}$ plus exogenous AsA/L-GalL application. IR29 and FL483 were grown in the same conditions as previously described. Plants in each container were first sprayed with 0.05% Trition-X100 to remove the hydrophobic layer on leaf surfaces, and rinsed with distilled water. For each plant, 5 ml of AsA or L-GalL solution (5 mM) was sprayed on the shoots in the afternoon once prior to the Fe treatment. Subsequently the spray application was repeated once a day after starting Fe treatment. After 4 days of Fe treatment, leaf bronzing symptoms were scored; shoots were harvested and flash-frozen in liquid N for further investigations. Additionally, two genotypes that were contrasting in Fe tolerance despite similar shoot Fe concentration (Matthus et al. 2015), Kiang-Chou-Chiu (KCC, sensitive genotype, *indica*) and Taichung Native 1 (TN1, tolerant genotype, *indica*) were investigated to further investigate the correlations between ascorbate recycling and Fe tolerance.

*3.3.5 RNA extraction and microarray slide hybridization*

Total RNA was isolated using a plant RNA extraction kits (for shoot samples, Peqlab, Erlangen, Germany; for root samples, Qiagen GmbH, Düsseldorf, Germany) following the manufacturer's instructions. Genomic DNA was removed using on-column RNAse-free DNAase (shoot, Peqlab; root, Qiagen).

Two replicates of root/shoot RNA samples in each treatment from both genotypes were first analyzed for RNA purity (Nanodrop 2000C, Thermo Fisher Scientific Inc., Wilmington, USA) and integrity (Bioanalyzer, Agilent Technologies, Santa Clara, USA). Samples with RNA integrity number > 8 were used for microarray slide hybridization (OakLabs GmbH, Hennigsdorf, Germany). Fifty ng of RNA were used to generate cRNA for one-color microarray slide hybridization. The 8 × 60K microarray slides (Agilent 60-mer SurePrint technology, Agilent Technologies) contained 59,336 *indica* rice specific probes that were designed based on the *indica* rice genome database from Beijing Genome Institute (BGI, http://rice.genomics.org.cn/). Since no gene annotations are available from the BGI database, all probe sequences were first mapped to the coding sequences (CDS) of *japonica* rice from MSU database

(http://rice.plantbiology.msu.edu) using MUMmer 3.0 (http://mummer.sourceforge.net/); then probe sequences were blasted against the BGI database (http://rise2.genomics.org.cn/page/rice/mapview.jsp) to obtain the BGI gene ID and gene ontology (GO) information. Raw fluorescence intensities were quantile-normalized with the software DirectArray from OakLabs using the ranked median quantiles according to Bolstad et al. (2003).

### *3.3.6 Quantitative reverse transcription PCR*

Three replicate samples of root/shoot RNA samples in each treatment from both genotypes were used for validation of microarray data by quantitative real-time PCR (qRT-PCR) in selected genes. Three hundred ng of shoot/root RNA were reverse-transcribed using the GoScript™ Reverse Transcription System (Promega GmbH, Mannheim, Germany). qRT-PCR was performed with the GoTaq® qPCR master mix (Promega) using a StepOne Plus Realtime PCR system (Applied Biosystems, Darmstadt, Germany). The reaction conditions were set up as follows: an initial denaturation step (10 min, 95°C), followed by 40 cycles of denaturation (15 sec, 95 °C) and annealing/extension (1 min, 60°C). Relative expression was calculated using the comparative ΔΔCT method with the expression level of IR29 in the control treatment as calibrator and 18S rRNA as the endogenous reference (Frei et al. 2010a). The amplification efficiency of all primers in this study was tested with serial dilutions of cDNA templates and was always >80%.

### *3.3.7 ROS staining in leaves*

*In-situ* detection of ROS in leaves was conducted according to Höller et al. (2014). In brief, the first and second youngest fully expanded leaves were detached followed by washing with 0.05 % Triton X-100 and rinsing with distilled water for three times. Then leaves were submerged in 0.5 mg $ml^{-1}$ 3,3'-Diaminobenzidine (DAB, pH 3.8) solution for 12 hours in the dark and afterwards rinsed again with distilled water. To remove chlorophyll, the leaves were incubated in a bleaching solution containing glycerol, lactic acid and ethanol at a ratio of 1:1:4, at 85°C for 1 h, and then stored in fresh bleaching solution. ROS formation was visualized as brown precipitation documented by a camera (550D, Canon Deutschland GmbH, Krefeld, Germany).

### *3.3.8 Fe distribution in different tissues*

The leaf blades were separated from the sheath and stem compartment (termed as SS in following text). Both tissues were dried in paper bags at 70 °C for three days and ground into a fine powder, followed by digestion with 4 ml 65 % $HNO_3$. The digested samples were then diluted to final volume of 25 ml. After filtration, Fe concentration was determined by atomic absorption spectrometry (Perkin-ELMER 1100B, Überlingen, Germany).

*3.3.9 Ferrous and ferric ion staining*

To investigate the cellular distribution of $Fe^{2+}$ and $Fe^{3+}$, two staining reagents were applied (Roschzttardtz et al. 2009; Engel et al. 2012b). Leaf blade, sheath and stem from the plants in both control and Fe treatment were detached and placed in 15 ml tubes containing 5 mM 2-2' bipydidyl ($Fe^{2+}$ staining), or 2% (w/v) potassium ferrocyanide with 2% (v/v) HCl (Perls reagent for $Fe^{3+}$ staining) for 4 h. Different tissues were sliced manually with razor blades for microscopy using a Leica DM LB light microscope (Leica Microsystems, Wetzlar, Germany), and photographed with a Leica DCF425 digital camera (Leica Microsystems, Wetzlar, Germany). In leaf blades, the green color of the chlorophyll interfered with the blue color of the $Fe^{3+}$ staining, leading to unclear images which are not presented.

*3.3.10 Ascorbic acid and malondialdehyde assay*

Reduced and oxidized AsA in shoots were measured according to Ueda et al. (2013). Briefly, around 80 mg of the samples were ground with liquid N and then dissolved in 1 ml 6% (w/v) metaphosphoric acid (MPA) containing 1 mM ethylene-diaminetetra-acetic acid (EDTA). Supernatants were collected after centrifuging the mixture at 15,000 g and 4°C for 20 min. Reduced AsA concentration was measured with 10 µl of the extracts added into the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0) and 0.1 units ascorbate oxidase (AO). Oxidized AsA was analyzed by mixing 10 µl of extracts to 80 µl of 100 mM potassium phosphate buffer (pH 7.8) with 10 µl of 4 mM dithiothreitol (DTT). Absorbance was monitored in a microplate reader (Powerwave XS2, BioTek Instruments, Inc., Winooski, USA) at 265 nm in the UV-transparent 96-well microplates until it was constant ($\varepsilon$ =14.3 $mM^{-1}$ $cm^{-1}$). Total AsA was calculated as the sum of reduced and oxidized AsA. Redox state was calculated as the percentage of reduced AsA in total AsA.

Malondialdehyde (MDA) was measured according to Höller et al. (2014). In brief, 100 mg of shoot material was dissolved in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) and the mixture was centrifuged at 14,000 g and 4 ºC for 15 min. Two aliquots of the same extract were mixed with reaction solution I (background reference) that contained 0.01% (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT) dissolved in 20% TCA (w/v), and reaction solution II additionally containing 0.65% 2-thiobarbituricacid (TBA), respectively. The mixture was incubated at 95 ºC for 30 min. After stopping the reaction on ice, mixture solutions were centrifuged (ten min, 8,000 g and 4 °C,) and absorbance was measured at 440, 532 and 600 nm with a microplate reader (Powerwave XS2, BioTek). Calculations of MDA concentrations included background corrections and correction factors for anthocyanin and unspecific turbidity.

*3.3.11 Enzymes activity assays*

Ascorbate oxidase (EC 1.10.3.3) activity was determined according to Pignocchi et al. (2003) with modifications. Around 100 mg of shoot samples were crushed with liquid N and mixed with 1 ml of 100 mM sodium phosphate buffer (pH 6.5). The mixture was then centrifuged at 10,000 g for 20 min at 4 ºC. The assay mixture contained 80 µL of 100 mM sodium phosphate buffer (pH 5.6), 10 µL of sample extract and ten µL of 2 mM reduced AsA. The kinetics was read at the wavelength of 265 nm ($\varepsilon$ = 14 $mM^{-1}$ $cm^{-1}$) for three min with a microplate reader (Powerwave XS2, BioTek). The ionically bound AO activities were also tested but found to be negligible.

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured according to Frei et al. (2012). Around 100 mg ground plant material was immersed in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, 2 mM EDTA and 1 mM $MgCl_2$. The mixture was centrifuged at 13,000 g for 10 min at 4 ºC. The assay mixture consisted of 70 µL of 50 mM potassium phosphate buffer (pH 6.5), 10 µL of sample extracts, 10 µL of 5 mM dehydroascorbic acid (DHA) and 50 mM reduced glutathione (GSH). The kinetics of the absorbance of AsA were recorded at 265 nm ($\varepsilon$ = 14 $mM^{-1}$ $cm^{-1}$) for 3 min. A blank without sample extract was added to correct for the non-enzymatic reduction of DHA.

Ascorbate peroxidase (APX, EC 1.11.1.11) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activities were measured using the same extracts following the protocol described in Frei et al. (2012). Around 100 mg of flash-frozen and ground leaf material was dissolved in 1 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM AsA and 1 mM EDTA. The extracting mixture was centrifuged for 30 min at 10,000 g and 4 °C. The reaction mix for APX activity contained 100 mM potassium phosphate buffer (pH 6.8), 0.6 mM AsA, 10 µl of 0.03 % $H_2O_2$, and 10 µl of enzyme extract. The oxidation of AsA was monitored at 290 nm for 30 s ($\varepsilon$ = 2.8 $mM^{-1}$ $cm^{-1}$). To determine MDHAR activity, the reaction mix contained 50 mM Tris-HCl buffer (pH 7.6), 0.1 mM nicotinamide adenine dinucleotide (NADH), 2.5 mM AsA, 0.1 units of AO and 10 µl of the plant extract. The oxidation of NADH was monitored at 340 nm for 3 min ($\varepsilon$ = 6.2 $mM^{-1}$ $cm^{-1}$). Protein concentrations in all enzyme extracts were measured according to Bradford (1976).

*3.3.12 Data processing and statistical analysis*

The significance of transcriptome data was tested using a mixed model in PROC MIXED (SAS 9.3, SAS Institute Inc., Cary, USA) with genotype, treatment and genotype by treatment interaction as fixed effects and replicate as random effect (Frei et al. 2010a). To avoid false significances declared due to multiple testing of a large number of probes, a false discovery rate adjustment of *P*-values was conducted

using the SAS MULTTEST procedure (Benjamini and Hochberg 1995). Transcripts were considered as differentially regulated if the *P*-value was <0.05, but transcripts with normalized intensity values <50 in all experimental conditions were not considered as significant.

For statistical analyses of other physiological data, the software SPSS was applied for analysis of variance and Pearson correlation (IBM SPSS Statistics 21, Ehningen, Germany). Post-hoc multiple comparisons for observed means were conducted using the LSD-test if appropriate.

## 3.4 Results

### *3.4.1 Stress symptoms in IR29 and FL483*

After four days of Fe treatment, visible symptoms on leaves were scored (Fig. 1A). IR29 showed significantly higher leaf bronzing score than FL483 (*P* <0.001) confirming its susceptibility to Fe treatment seen in our previous study (Wu et al. 2014). ROS formation in leaves of IR29 and FL483 were visualized using DAB staining (Fig. 1B). More abundant brown precipitates were observed in the leaves of IR29 than of FL483, which indicates more ROS formation in the sensitive line IR29.

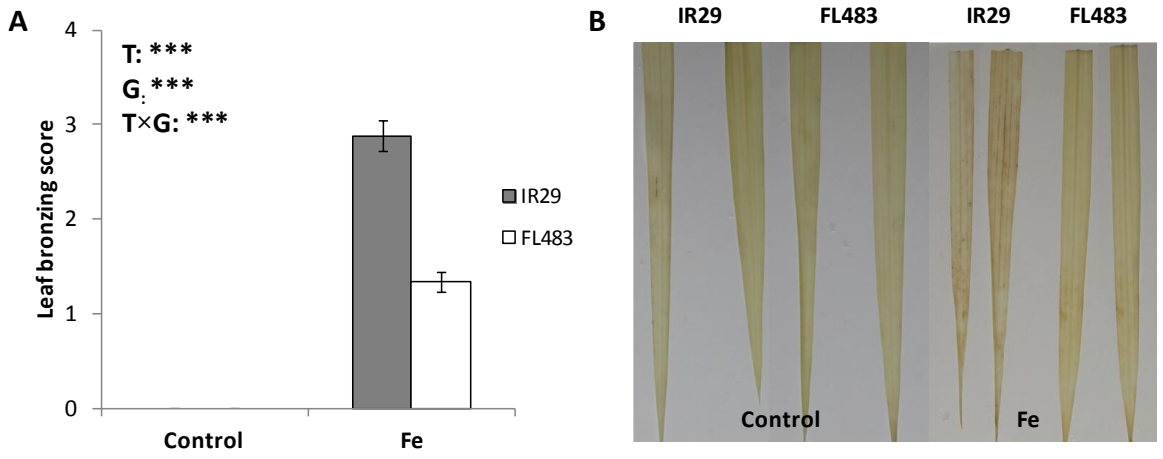


Figure 1. Stress response of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1000 ppm $Fe^{2+}$ for four days) (A) Leaf bronzing score of IR29 and FL483 (B) Hydrogen peroxide staining using 3,3'-Diaminobenzidine. Bars represent mean value ± standard error (n=6). T, treatment; G, genotype; *, *P* <0.05; ***, *P* <0.001; n.s, not significant.

### *3.4.2 Transcript profiling and validation using qRT-PCR*

To investigate the genome-wide transcript profile, we conducted microarray analyses and obtained the gene annotations from the MSU database and GO information from the BGI database. All probes with signal intensity values <50 were excluded in the subsequent analyses. After removing the probes that were not specifically assigned to single genes according to either the MSU or BGI database, 24,381 transcripts representing 22,950 MSU gene models and 1431 BGI specific gene models were expressed in roots. A total of 2034 transcripts showed significant down-regulation and 1656 transcripts were up-regulated in the Fe treatment (Table 1). In shoots, 25,132 probes representing 23,659 gene models in MSU database and 1,443 specific gene models in the BGI database were expressed. In total, 195 transcripts were significantly down-regulated while 645 transcripts were up-regulated (*P* <0.05) in the Fe treatment. A complete dataset of transcript regulation in shoots and roots is provided in Supporting Information Data S1.

The aim of this study was to identify genotypic differences between IR29 and FL483 related to Fe tolerance. In roots, 345 transcripts were differentially regulated between these two genotypes ($P$ <0.05). In the tolerant genotype FL483, 154 transcripts showed down-regulation, while 191 transcripts showed up-regulation compared to IR29 (Table 1). However, none of the genes showed any significant treatment by genotype interactions in roots. In shoots, 605 transcripts were differentially regulated between the two genotypes ($P$ <0.05). Among those, 434 transcripts were down-regulated and 171 transcripts up-regulated in FL483. A total number of 94 genes showed significant treatment by genotype interactions (Table 1).

The genome-wide transcript profiling from the microarray experiment was validated by qRT-PCR using a subset of nine genes in shoots and seven genes in roots (complete information of gene expression pattern and primers see Supporting Information Data S2). The subset represented genes showing significant treatment and /or genotype effects with a broad range of signal intensities from <140 to >65,000. The microarray experiment and qRT-PCR analysis were in good agreement ($R^2$=0.9084, Supporting Information Figure S1).

Table 1. Genome-wide transcripts profiling in shoot and root from microarray experiment

| Tissue | Total transcripts | Treatment (Fe/Control) | | Genotype (FL483/IR29) | | Treatment by genotype interaction |
|---|---|---|---|---|---|---|
| | | Down | Up | Down | Up | |
| Root | 24381 | 2304 | 1656 | 154 | 191 | - |
| Shoot | 25132 | 195 | 645 | 434 | 171 | 94 |

The numbers indicate the genes that were differentially regulated in two treatments and in two contrasting genotypes. The complete list of genes used for generating this table is given in Supporting Information Data S1. A $P$-value <0.05 after false discovery rate correction was considered as significant.

*3.4.3 Hypothesis I: Fe uptake, partitioning and speciation*

In the transcriptional regulation of genes related to different hypotheses, we differentiated between treatment responses in roots (Fig. 2) and shoots (Fig. 3), and differential expression between genotypes (Fig. 4). Regarding the genes involved in Fe uptake, we only focused on roots (Fig. 2). In Fe uptake Strategy I, 66 genes including $H^+$-ATPase, phenolics efflux transporters, Fe-chelate reductase and metal cation transporters were analyzed. Seven genes were significantly down-regulated by the Fe treatment. Ferric reductase genes (LOC_Os08g35210, LOC_Os09g26660, LOC_Os04g36720, LOC_Os04g48930) did not show any genotypic or treatment effect. However, the expression levels of six genes encoding metal cation transporters were significantly lower in the Fe treatment. One gene (LOC_Os03g29850) also showed a significant genotypic effect with a two-fold higher expression level in the tolerant line FL483 (Fig. 4). Out of 26 genes involved in Fe uptake Strategy II, only three showed significantly lower expression in the Fe treatment and no genotypic differences were observed. For Fe transporter genes in roots, eight ABC transporter family genes, two natural resistance-associated macrophage protein (NRAMP) and eight multidrug and toxic compound extrusion (MATE) efflux family protein genes were significantly regulated by Fe treatment. Two vacuolar iron transporter (*OsVIT*) genes were not differentially regulated either in different treatments or genotypes. Two ferritin genes involved in Fe storage were up-regulated about 4-fold in the Fe treatment but no genotypic differences were seen.

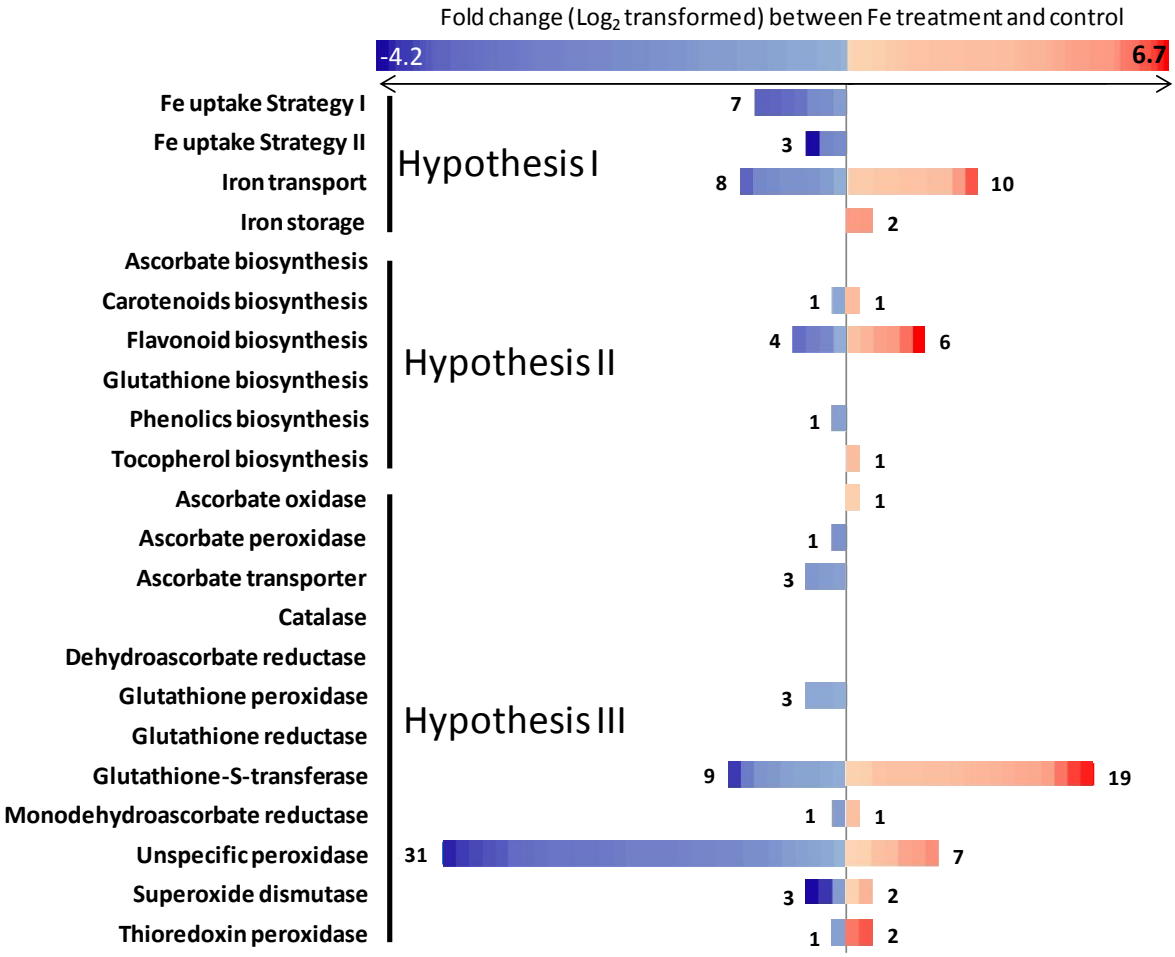


Figure 2. Effects of a four-day Fe stress (1,000 ppm) on the transcriptional regulation of root genes involved in different pathways in two rice genotypes IR29 and FL483 (N=8). The numbers next to the bars indicate how many genes were significantly regulated ($P$ <0.05). Red color indicates higher expression while blue colors indicates lower expression in Fe treatment compared to the control, and color intensity indicates the fold-change. A complete dataset is provided in the Supporting Information Data S3-S5.

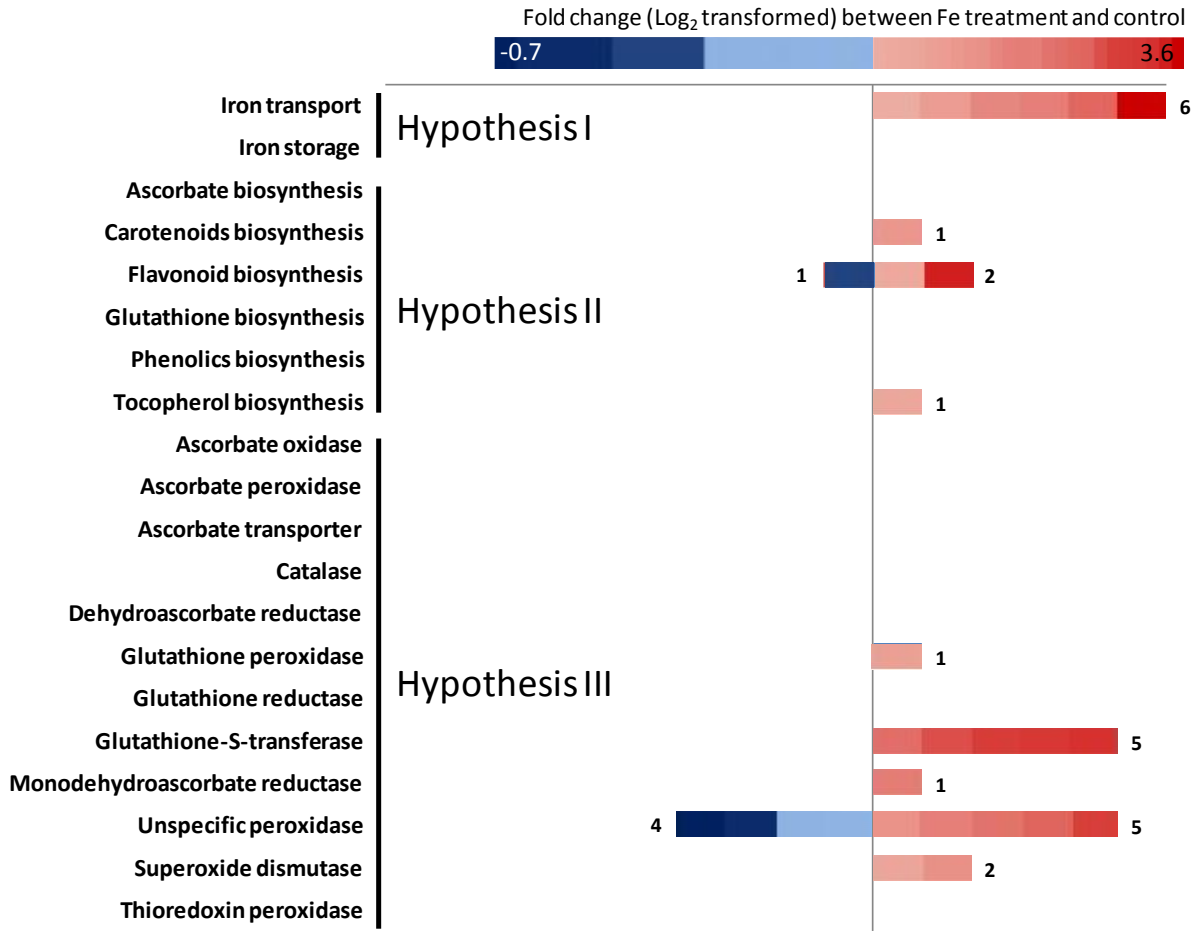


Figure 3. Effects of a four-day Fe stress (1,000 ppm) on the transcriptional regulation of shoot genes involved in different pathways in two rice genotypes IR29 and FL483 (N=8). The numbers next to the bars indicate how many genes were significantly regulated ($P$ <0.05). Red color indicates higher expression while blue colors indicates lower expression in Fe treatment compared to the control, and color intensity indicates the fold-change. A complete dataset is provided in the Supporting Information Data S3-S5.

In shoots, we only analyzed Fe transport and storage-related genes (Fig. 3). Four ATP-binding cassette (ABC) transporter family genes and two MATE efflux family protein genes were significantly up-regulated in the Fe treatment. LOC_Os01g50100 (ABC transporter family gene) and LOC_Os10g20350 (MATE efflux family gene) showed significant treatment, genotypic and interaction effects. Both genes were highly expressed in IR29 (Fig. 4). No treatment or genotypic significance was observed in either vacuolar Fe transporter or ferritin gene expressions. The complete dataset of the expression patterns of genes for Fe uptake, transport and storage can be found in Supporting Information Data S3.

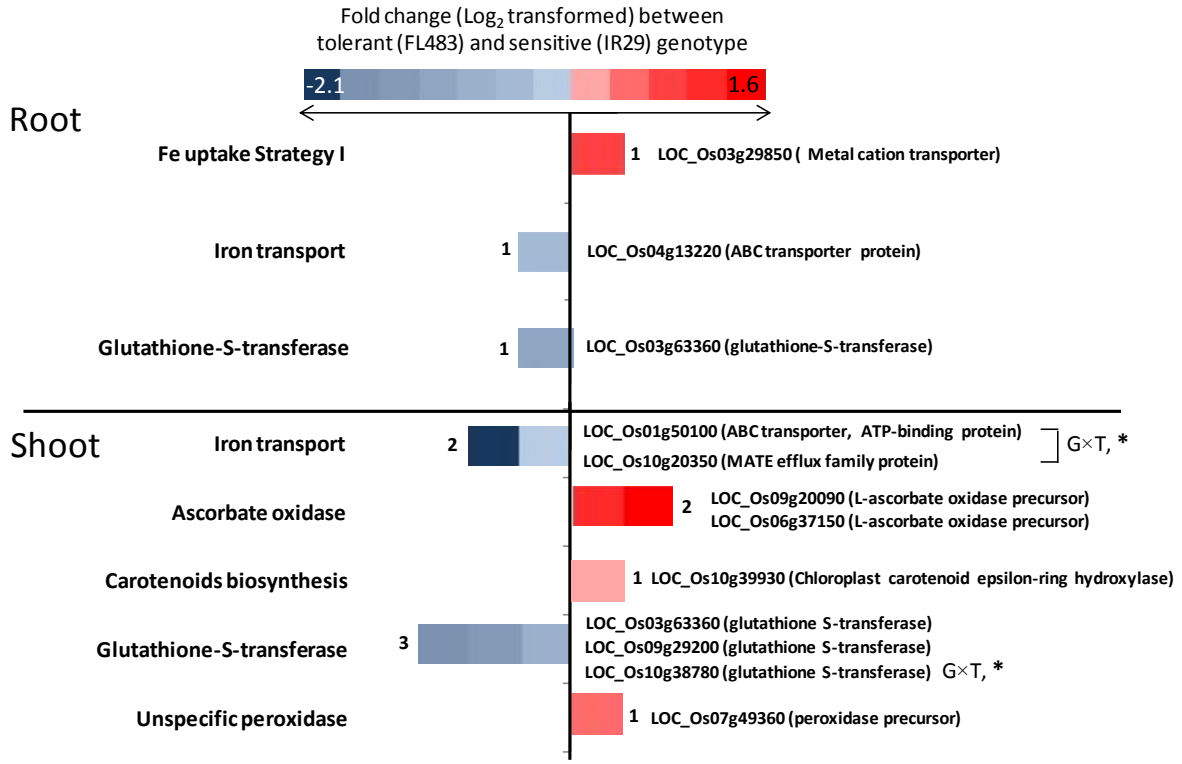


Figure 4. Differentially expressed genes between contrasting rice genotypes IR29 (sensitive) and FL483 (tolerant) after of a four-day Fe stress (1,000 ppm) (N=8 in both roots and shoots). The numbers next to the bars indicate how many genes were regulated ($P$ <0.05). Red color indicates higher expression while blue color indicates lower expression in tolerant genotype FL483 compared to the sensitive genotype IR29. The genes showing significant treatment by genotype interaction ($P$ <0.05) are indicated as: G×T, *****. A complete dataset is provided in the Supporting Information Data S3-S5.

Fe concentrations were measured in leaf blade and SS (sheath and stem compartment) to investigate Fe distribution at tissue scale (Fig. 5). In both genotypes, the Fe treatment significantly increased Fe concentrations in both shoot compartments ($P$ <0.001). As opposed to the control treatment, Fe concentrations under Fe toxicity were higher in the SS than in leaf blade (tissue by treatment interaction, $P$ <0.05), indicating that the plants retained more Fe in SS. However, no genotypic differences were observed in Fe partitioning at tissue scale suggesting that this mechanism was not underlying the contrasting tolerance between IR29 and FL483.

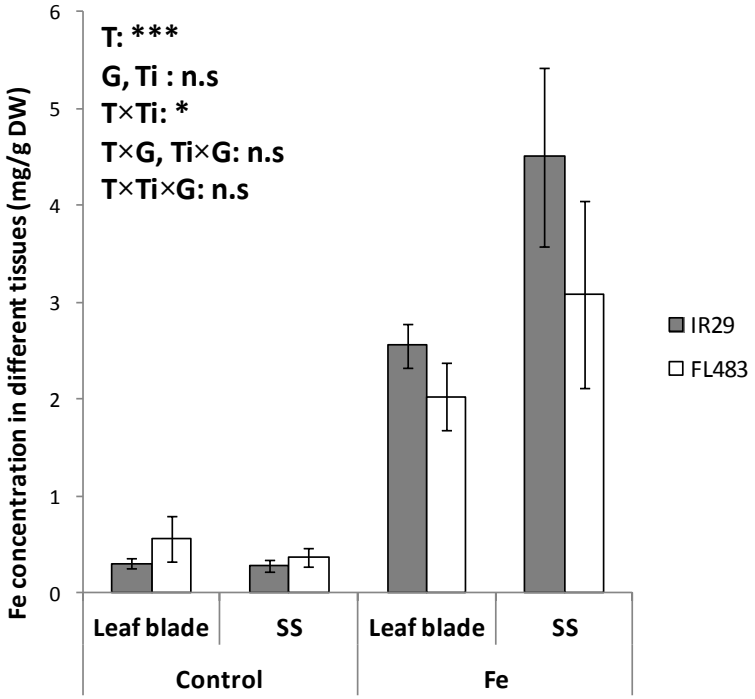


Figure 5. Fe concentrations in different tissues of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity. Bars represent mean values ± standard error (n=6). SS, leaf sheath and stem; T, treatment; G, genotype; Ti, tissue; *, $p$<0.05; ***, $p$<0.001; n.s, not significant.

Specific staining solutions were applied to differentiate visually between the different redox states of Fe in different tissues and contrasting genotypes. $Fe^{3+}$ in the internodes was localized in the fundamental parenchyma (FP) cells in or near vascular bundles in both genotypes (Fig. 6A). In the Fe treatment, $Fe^{3+}$ was highly accumulated in FP and xylem vessel cells in the internodes. $Fe^{2+}$ was localized in the same parts of the internodes except for the xylem vessels (Fig. 6B).

In leaf sheaths and leaf blades, chlorophyll interfered with the blue staining of $Fe^{3+}$. Therefore only results from $Fe^{2+}$ staining are shown. The localization of $Fe^{2+}$ in leaf sheath and blade were identical, i.e. in the upper and lower epidermis and the phloem. However, the two genotypes did not show obvious differences in the Fe distribution at the cellular level (Fig. 6C,D).

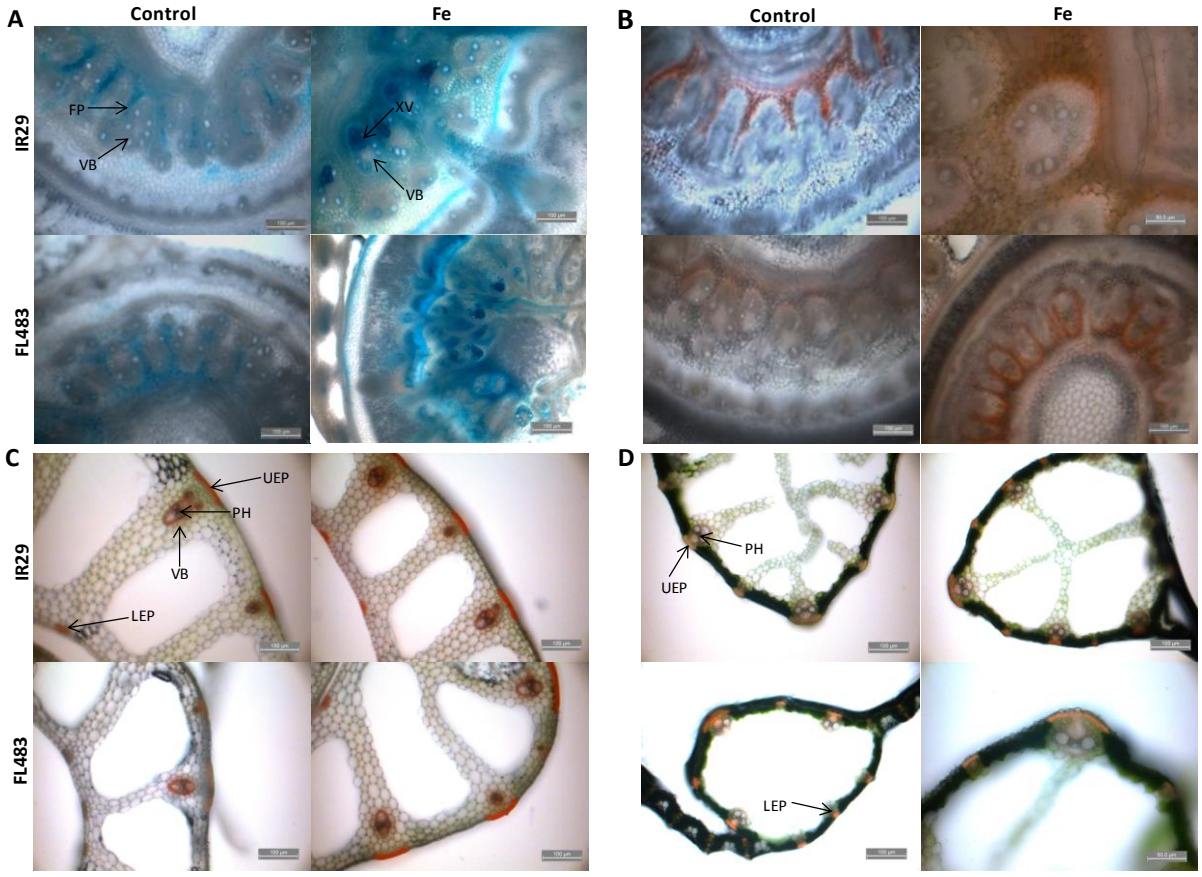


Figure 6. Fe staining in different tissues of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1,000 ppm $Fe^{2+}$ for 4 days). $Fe^{2+}$ and $Fe^{3+}$ ions were stained with 2-2'-bipyridyl and Perls reagents, respectively. (A) $Fe^{3+}$ staining in the first internode, (B) $Fe^{2+}$ staining in the first internode, (C) $Fe^{2+}$ staining in leaf sheath, (D) $Fe^{2+}$ staining in leaf blade. FP, fundamental parenchyma; VB, vascular bundle; XV, xylem vessel; UEP, upper epidermis; LEP, lower epidermis; PH, phloem.

### *3.4.4 Hypothesis II: Biosynthesis of antioxidants for ROS scavenging*

Genes involved in the biosynthesis of common antioxidants including ascorbic acid, glutathione, phenolics, tocopherol and carotenoids were analyzed. In roots, ten flavonoid biosynthesis genes were significantly regulated by Fe treatment. Among those, six genes were up-regulated and four genes were down-regulated ($P$ <0.05) (Fig. 2). The most pronounced up-regulation in the Fe treatment (>100-fold) was observed in a gene encoding a chalcone and stilbene synthases (LOC_Os07g34190). However, only few genes related to antioxidants biosynthesis were differentially regulated in shoots (Fig. 3). Only three flavonoid, one tocopherol and one carotenoid biosynthesis gene showed a significant treatment effect, and one carotenoid related gene, LOC_Os10g39930 (cytochrome p450 97B2) was significantly higher expressed in FL483 (Fig. 4). None of the genes involved in

antioxidants biosynthesis showed a significant treatment by genotype interaction in either roots or shoots. The complete dataset of the expression patterns of the genes for antioxidants biosynthesis can be found in Supporting Information Data S4.

### *3.4.5 Hypothesis 3: Antioxidant enzymes for ROS scavenging and antioxidants turnover*

Superoxide dismutase (SOD) genes with different co-factors showed contrasting responses to Fe treatment in roots (Fig. 2). Three Mn-SOD genes were significantly down-regulated while two Cu/Zn-SOD genes were up-regulated in the Fe treatment. Unspecific peroxidase (POX) genes are abundant in the rice genome. In roots, 38 POX genes were responsive to the Fe treatment. Among those, 32 genes were down-regulated while only six genes were up-regulated by Fe treatment. Glutathione-S-transferases (GST) were represented by 78 genes. A total of 19 GST genes were up-regulated while only nine were down-regulated in the Fe treatment (Fig. 2). Among these antioxidant enzymes, only one GST gene (LOC_Os03g63360) showed a significant genotypic effect in root and was expressed about two-fold higher in IR29 (Fig. 4).

Compared to the roots, fewer antioxidant enzymes showed significant responses to Fe in shoot. The expression of two Cu/Zn-SOD genes, one MDHAR gene and one glutathione peroxidase (GPX) gene were significantly induced by Fe treatment (Fig. 3). Out of the 100 POX genes with detectable expression in shoots, only ten genes showed a significant treatment effect. Similar results were observed in 73 GST genes, among which only six genes were induced by Fe treatment. Even though fewer genes were Fe responsive in shoots than in roots, more genes showed significant genotype effects (Fig. 4). A putative peroxidase precursor (LOC_Os07g49360) was expressed 1.8-fold higher in tolerant genotype. Three GST genes (LOC_Os10g38780, LOC_Os09g29200 and LOC_Os03g63360) were expressed about 1.9- to 2.3-fold higher in the sensitive genotype IR29. The expression of LOC_Os10g38780 also showed a significant treatment by genotype interaction. In ascorbate oxidase (AO) genes, LOC_Os09g20090 and LOC_Os06g37150 showed about 2.5- and 3.1-fold higher expression ($P$ <0.05) in the tolerant genotype FL483 than in IR29, respectively (Fig. 4). A complete dataset of the gene expressions in ROS scavenging and antioxidant-related enzymes is shown in Supporting Information Data S5.

Several genes involved in antioxidant enzymes showed genotypic differences (Fig. 4), including an AO gene (LOC_Os06g37150) which was previously found to be crucial in determining AO activity in rice (Ueda et al. 2015). Therefore we hypothesized that ascorbate turnover and especially AO activity might be involved in the hypothetical tolerance mechanisms and measured related enzyme activities. The

expression pattern of LOC_Os06g37150 (measured independently by qRT-PCR) was reflected in the AO enzyme activity, i.e. both genotypes showed higher expression in the Fe treatment and the tolerant genotype FL483 showed significantly higher (*P* <0.001) transcription than IR29 (Fig. 7A). The AO activity was not induced by the Fe treatment, while the genotypic difference was consistent with the gene expression pattern (Fig. 7B). DHAR activity was significantly induced by the Fe treatment (*P* <0.001) and IR29 showed higher DHAR activity than FL483 (*P* <0.001) (Fig. 7C). MDHAR activity was not significantly regulated in different treatments or genotypes (Fig. 7D).

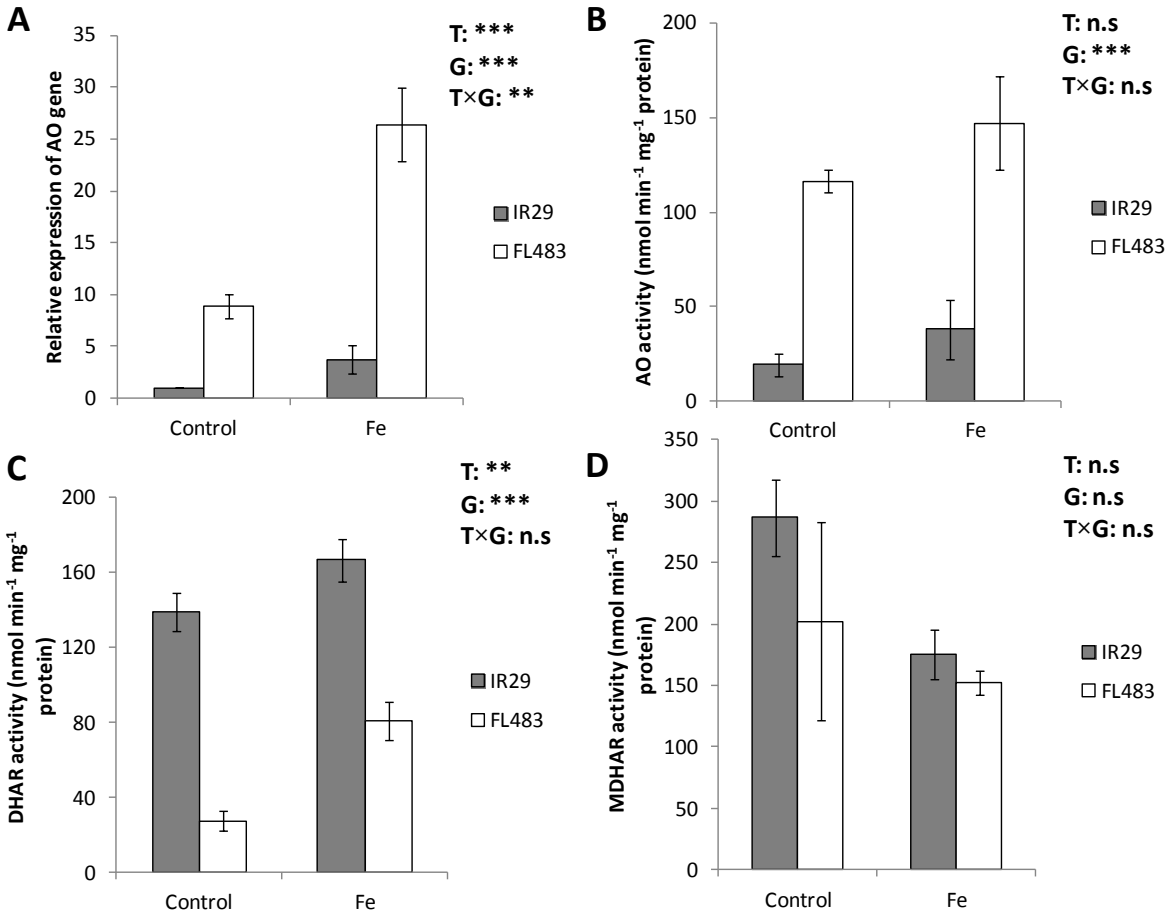


Figure 7. Gene expression and enzymatic activities of ascorbate-related enzymes in shoots of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1,000 ppm $Fe^{2+}$ for 4 days). (A) Relative gene expressions of ascorbate oxidase (AO) gene LOC_06g37150, which dominates *in vivo* AO activity in rice (Ueda *et al.*, 2015). (B) Ascorbate oxidase activity, (C) Dehydroascorbate reductase activity and (D) Monodehydroascorbate reductase activity. Bars represent mean values ± standard error (n=3). T, treatment; G, genotype; **, *P* <0.01; ***, *P* <0.001; n.s, not significant.

*3.4. 6 Hypotheses testing experiments*

The transcriptomic and enzymatic activity results demonstrated that the tolerant genotype showed lower DHAR but higher AO activity than the sensitive. Thus we hypothesized that reduced AsA might not favor the tolerance to Fe toxicity but rather aggravate the oxidative stress in the presence of high levels of $Fe^{2+}$. To test this hypothesis, we studied the effect of exogenous reduced AsA and L-GalL application on Fe toxicity tolerance. Exogenous AsA and L-GalL application significantly raised the foliar concentration of reduced and total AsA in both genotypes under both control and Fe conditions (Fig. 8A, B; Fig. 9A, B). Fe treatment had no effect on the reduced and total AsA concentration in shoots. Both AsA and L-GalL applications under control conditions did not significantly affect the AsA redox state. However, under Fe toxic conditions, both genotypes showed significantly lower AsA redox state when supplied with exogenous AsA (Fig. 8C) but not L-GalL (Fig. 9C). Fe treatment reduced the redox state in both genotypes ($P$ <0.05). Moreover, the sensitive genotype IR29 showed consistently higher ($P$ <0.05) AsA redox state than the tolerant genotype FL483 (Fig. 8C; Fig. 9C). As a biomarker for lipid peroxidation, shoot MDA concentrations that positively correlated with leaf bronzing symptoms (Supporting Information Figure S3), were markedly induced by Fe treatment in both genotypes. However, FL483 showed significantly lower ($P$ <0.01) MDA concentration in the Fe treatment than IR29. The application of either AsA or L-GalL had no significant effect on the shoot MDA concentrations in the absence of excess Fe. However, both genotypes showed significantly higher MDA concentration in Fe stress when provided with exogenous AsA or L-GalL compared to the Fe treatment alone (Fig. 8D; Fig. 9D). Both AsA and L-GalL applications did not affect DHAR and AO activities in either control or Fe treatment (Fig. 8E,F; Fig. 9E,F). Consistent with the previous experiment, the Fe treatment markedly induced the DHAR activity ($P$ <0.01) in both genotypes, and IR29 showed significantly higher DHAR activity and lower AO activity than FL483 ($P$ <0.001).

To test whether the link between ascorbate metabolism and tolerance to Fe toxicity was genotype specific or more generally applicable, another contrasting pair of genotypes (sensitive genotype KCC, tolerant genotype TN1) was investigated, which was selected from a global rice population previously screened under Fe stress (Matthus *et al*. 2015). KCC showed significantly higher leaf bronzing score (Supporting information Fig. S4A) and shoot MDA concentration (Supporting information Figure S4B). DHAR activity in shoots was enhanced by Fe treatment while AO activity was not affected (Supporting information Figure S4C,D). Consistent with our previous results, genotypic differences were observed in shoot DHAR and AO activity, i.e. the tolerant genotype possessed lower DHAR activity and higher AO

activity than the sensitive genotype, although these differences were less pronounced than those between IR29 and FL483.

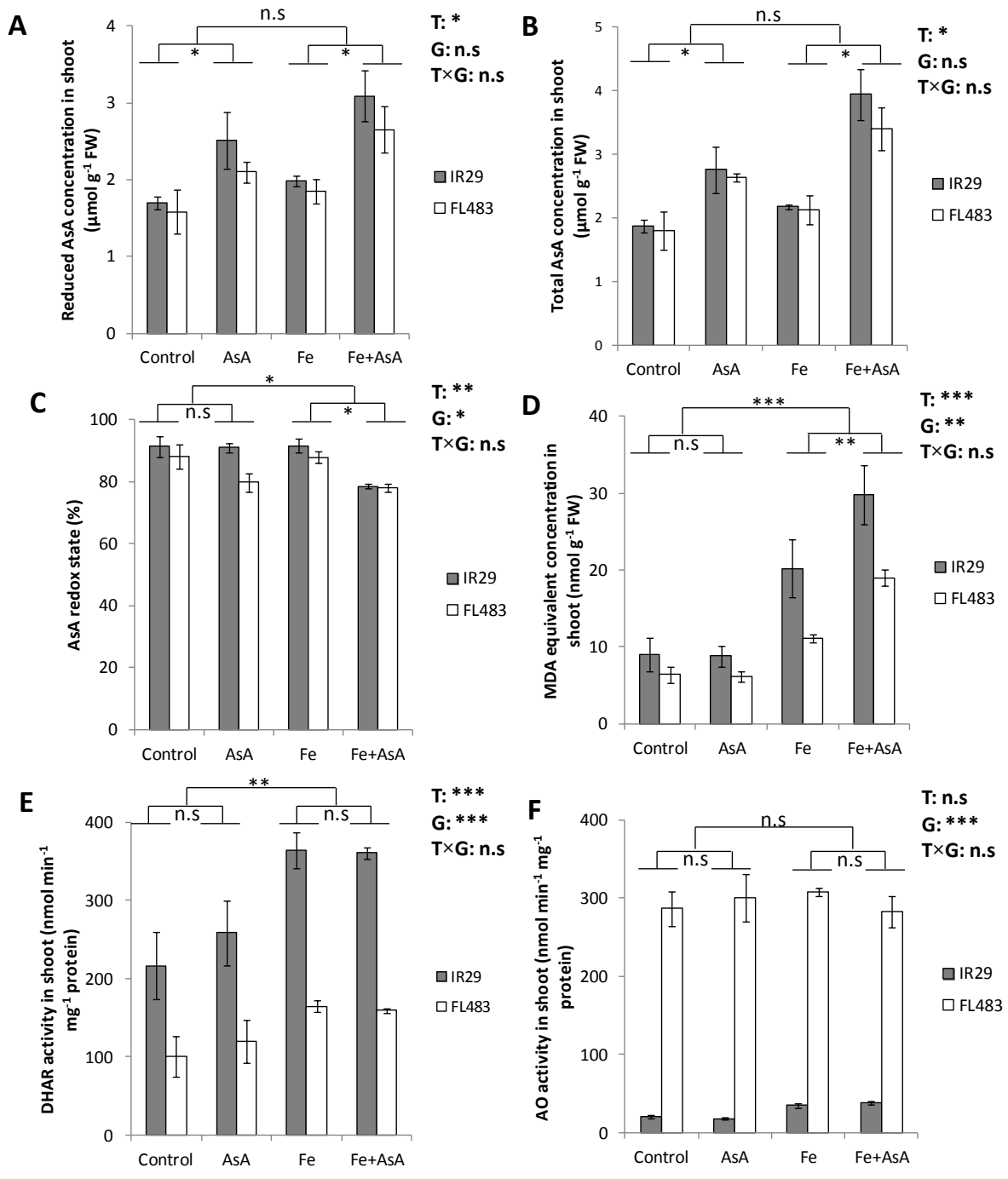


Figure 8. Effect of exogenous reduced ascorbic acid (AsA) application on lipid peroxidation in shoots of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1,000 ppm $Fe^{2+}$ for 4 days). Plants were exposed to four treatments including control, 5 mM exogenous AsA spray, Fe toxicity alone and Fe toxicity with AsA spray. (A) Reduced AsA concentration, (B) total AsA concentration (C) AsA redox state, (D) malondialdehyde (MDA) concentration, (E) Dehydroascorbate reductase activity, (F) Ascorbate oxidase activity in shoot were shown. Bars represent mean values ± standard error (n=4). Post-hoc multiple comparison was conducted with LSD method. T, treatment; G, genotype; *, *P* <0.05; **, *P* <0.01; ***, *P* <0.001; n.s, not significant.

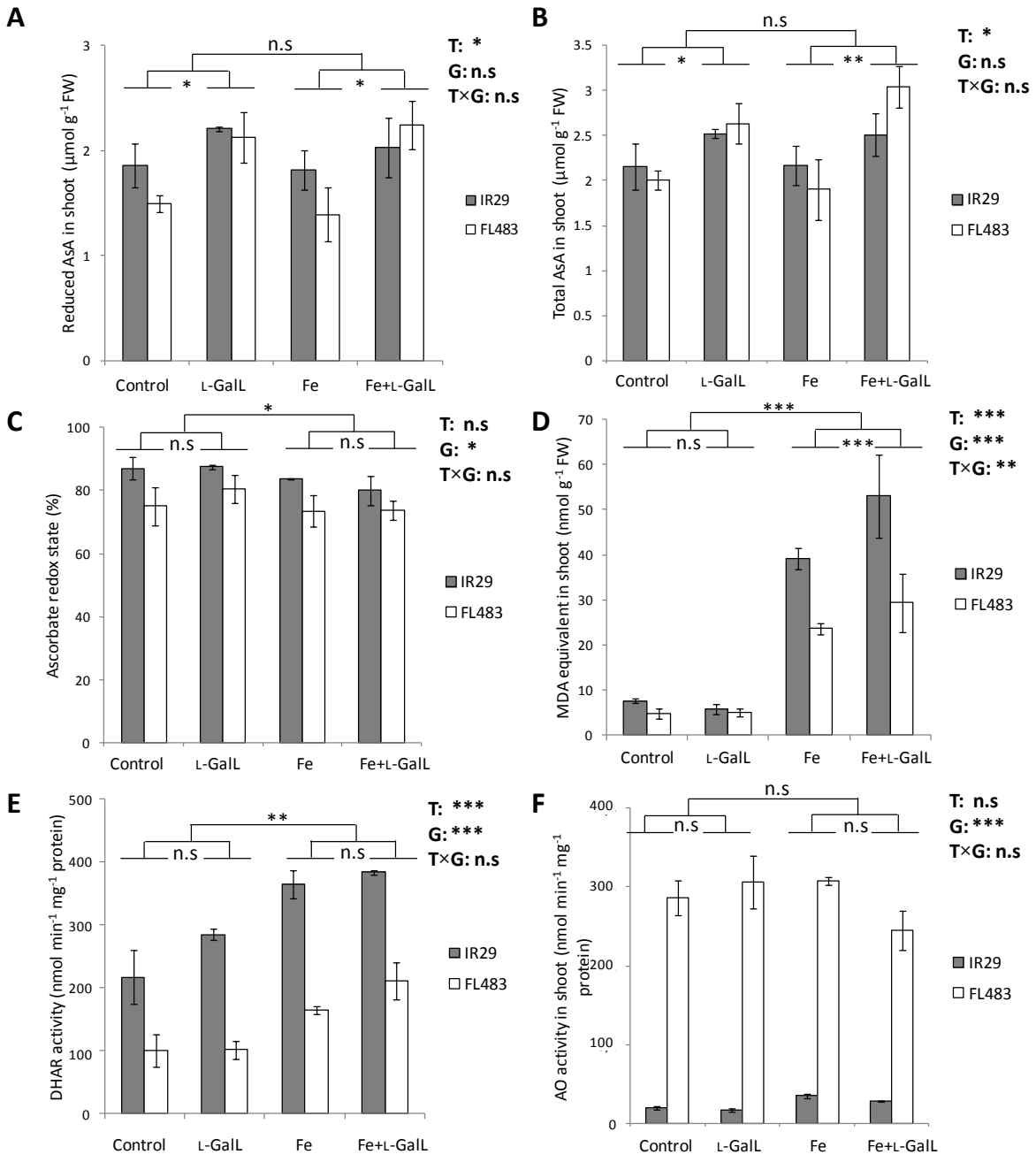


Figure 9. Effect of exogenous L-galactono-1,4-lactone (L-GalL) application on lipid peroxidation in shoots of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1,000 ppm $Fe^{2+}$ for 4 days). Plants were exposed to four treatments including control, 5 mM exogenous L-GalL spray, Fe toxicity alone and Fe toxicity with L-GalL spray. (A) Reduced AsA concentration, (B) total AsA concentration (C) AsA redox state, (D) malondialdehyde (MDA) concentration, (E) Dehydroascorbate reductase activity, (F) Ascorbate oxidase activity in shoot were shown. Bars represent mean values ± standard error (n=4). Post-hoc multiple comparison was conducted with LSD method. T, treatment; G, genotype; *, $P$ <0.05; **, $P$ <0.01; ***, $P$ <0.001; n.s, not significant.

## 3.5 Discussion

When exposed to excess Fe, more genes were significantly regulated in roots than in shoots (Table 1). These results are in agreement with the findings by (Quinet et al. 2012), where rice plants were exposed to 125 ppm $Fe^{2+}$ for three days. However, a much larger number of genes was significantly regulated in our study than in the study by (Quinet et al. 2012), presumably due to a higher concentration of $Fe^{2+}$ (1,000 ppm), which aimed at simulating an acute Fe shock rather than a chronic stress. Despite the large number of genes significantly responding to Fe stress, fewer genes showed genotypic differences in expression in the roots compared to the shoots, and no significant treatment by genotype interactions occurred (Table 1). This is in agreement with our assumption that differences in tolerance between IR29 and FL483 can be explained with a shoot-based rather than a root-based mechanism. FL483 carries tolerant alleles at two previously detected QTLs (*qFETOX-1-1* and *qFETOX-1-2*) (Wu et al. 2014) and was classified as a tolerant includer, i.e. it is significantly more tolerant to Fe toxicity than IR29 (Fig. 1) despite similar shoot Fe concentration (Fig. 5). The high number of significantly regulated transcripts in roots is therefore likely to represent stress responses rather than tolerance mechanisms. We also analyzed the expression levels of genes located 1 Mb up- and down-stream of the two QTLs (Supporting Information Data S6). These regions do not contain very obvious candidate genes for iron toxicity tolerance based on their annotation and expression pattern. This could be due to the fact that genes underlying QTLs were often found to have regulatory function, (e.g. a QTL for grain width and weight, GW2, (Song et al. 2007)), were not present in reference genomes at all (e.g. phosphorus-starvation-tolerance 1, (Gamuyao et al. 2012)), or carried functional mutations rather than showing differences in gene expression (e.g. the blast disease resistant gene, Pi-21, (Fukuoka et al. 2009)). Therefore further mapping and sequencing of candidate regions would be required to nominate candidate genes. However, the objective of this study was not to identify candidate genes underlying the previously mapped QTLs, but rather to elucidate shoot-based tolerance mechanisms to Fe toxicity, for which the contrasting genotype pair IR29 and FL483 provides an ideal model system, because they differ significantly in Fe tolerance, but not in Fe uptake and plant morphology. Three specific hypotheses were addressed.

### *3.5.1 Hypothesis I: Fe uptake, partitioning and speciation*

We analyzed expression patterns and responses of genes involved in Fe uptake (Fig. 2). In Fe deficient conditions, ferric reductases play an important role in taking up Fe efficiently as part of the Strategy I (Ishimaru et al. 2007). However, under excess $Fe^{2+}$ conditions, rice plants do not require ferric reductase to generate soluble $Fe^{2+}$, which explains why ferric reductase gene expressions did not show any treatment effect. In

contrast to Fe deficient conditions (Zheng et al. 2009), excess $Fe^{2+}$ suppressed the expression of metal cation transporter genes possibly involved in Strategy I Fe uptake. Regarding Fe uptake Strategy II, phytosiderophore synthetases and Fe-phytosiderophore transporters (OsYSL16) were down-regulated in the excess Fe treatment, which is in agreement with the findings by Quinet et al. (2012). No genotypic differences occurred in the expression of Fe uptake genes (except for one metal cation transporter gene, LOC_Os03g29850 that showed even higher expression in the tolerant genotype), which is in agreement with our hypothesis that tolerance in FL483 is conferred via a shoot-based mechanism. Regarding the sub-cellular Fe distribution, vacuoles were previously reported to absorb major fractions of Fe in Arabidopsis (*Arabidopsis thaliana*) embryo cells (Roschzttardtz et al. 2009). However, two putative vacuolar Fe transporter genes (LOC_Os04g38940 and LOC_Os09g23300) showed only significant treatment effects in roots but were not differentially regulated between the two genotypes, indicating that the storage of excess Fe vacuoles was not linked to the tolerance in FL483. The same was true for genes encoding the Fe storage protein ferritin, which was previously proposed to confer tolerance to Fe toxicity (Stein et al. 2009). Organ-specific Fe partitioning, e.g. leaf sheath and stem was investigated as one aspect of shoot tolerance by Engel et al. (2012a), presuming that storing Fe in less photosynthetically active tissues limits the generation of ROS. However, since no genotypic differences were found in the tissue-specific Fe levels (Fig. 5 and Fig. 6), the hypothesis of Fe uptake, transport, and partitioning being involved in tolerance of FL483 was rejected.

### *3.5.2 Hypothesis II: Biosynthesis of antioxidants scavenging ROS*

Ascorbic acid (AsA) is the most abundant water-soluble antioxidant in plants (Gallie 2013). It can protect rice plants from abiotic stresses such as Zn deficiency (Frei et al. 2010b; Höller et al. 2015) and ozone stress (Frei et al. 2012), and it exchanges electrons with its sister antioxidant glutathione via the ascorbate glutathione cycle (Noctor and Foyer 1998). In this study, none of the genes involved in AsA or glutathione biosynthesis showed any significant treatment or genotype effects. We concluded that the biosynthesis of AsA was not associated with tolerance in the pair of genotypes investigated in this study. In agreement with Höller et al. (2015), total ascorbate level was not significantly correlated with the oxidative damage caused by Fe toxicity in rice. In roots, genes involved in the biosynthesis of other antioxidants, e.g. phenolics, flavonoid, tocopherol and carotenoids, were significantly affected by the Fe treatment, but no significant genotypic or interaction effects occurred. Carotenoids are natural isoprenoid pigments with antioxidant activity (Cazzonelli 2011). We observed one gene for carotenoids biosynthesis showing higher expression in tolerant genotype (LOC_Os10g39930, Fig. 4). However, carotenoids

were reported to react only with singlet oxygen ($^1O_2$), but they cannot detoxify radicals such as superoxide, hydroperoxides or hydroxyl (Chaudière and Ferrari-Iliou 1999; Gill and Tuteja 2010), making it less likely that this gene is directly associated with tolerance in FL483.

*3.5.3 Hypothesis III: ROS scavenging enzymes and antioxidants related enzymes*

Contradictory results were reported in the literature regarding SOD activities under Fe toxic conditions in rice. Fang et al. (2001) detected decreased SOD activity in detached rice leaves treated with 10 mM $FeSO_4$, while increased SOD activities were found in leaf sheaths treated with 8.9 mM $FeSO_4$ by Majerus et al. (2007). In this study, we observed different responses of SOD genes under Fe toxic conditions (Fig. 2; Fig. 3), which can be explained by different co-factors functioning in SOD enzymes and their sub-cellular localization. Mn-SOD is typically located in the mitochondria and peroxisomes, while Cu/Zn-SOD is located in chloroplast and cytosol (Gill and Tuteja 2010). These results suggest that different cell components respond differently to excess Fe.

Although one POX gene (LOC_Os07g49360, Fig. 4) showed higher expression in the tolerant genotype, the POX activities measured in an enzymatic assay only showed a significant treatment effect without genotypic differences (Supporting Information Figure S2.A). Plant GSTs are a large gene family with multiple functions in plants, including DHAR activity, which may be related to the tolerance to abiotic stresses (Liu et al. 2013). The three GST genes showing genotypic differences in shoots (Fig. 4) were classified into the *tau* (τ) class (Kumar et al. 2013). Over-expression of *tau* class of GSTs in transgenic tobacco plants increased the tolerance to abiotic stresses e.g. herbicide exposure or salt stress (Karavangeli et al. 2005; Jha et al. 2011). However, lower expression of these GSTs was found in the tolerant genotype (Fig. 4), thus raising the question whether the expression of these GST genes is associated with DHAR activity measured in an enzymatic assay (Fig. 7C), which showed the same pattern. Further investigations using mutant lines of these genes are warranted. One gene encoding an AO (LOC_06g37150, Fig. 7A) showed significantly higher expression in the tolerant line FL483. The expression of this gene was highly correlated with AO activity measured in rice shoots previously (Ueda et al. 2015), which was confirmed by the AO activity data obtained in our study (Fig. 7B). Together with the lower DHAR activity detected in FL483, these data imply that a high rate of AsA reduction was associated sensitivity, while AsA oxidation was associated with tolerance.

Based on these results, we hypothesized that reduced AsA may act as a pro-oxidant rather than antioxidant in Fe toxic conditions. In fact, several previous studies suggested the pro-oxidant property of AsA in vitro (Halliwell 1996; Rietjens et al.

2002; Yen et al. 2002) due to the interaction with transition metal ions, such as $Fe^{2+}$, $Cu^{+}$ (Halliwell 1996). However, the biological relevance of AsA pro-oxidant effects in planta remains unclear. Indeed our experiment, in which exogenous reduced AsA was provided to IR29 and FL483 thus enhancing lipid peroxidation, confirmed such a pro-oxidant activity in rice plants grown in Fe toxic conditions (Fig. 8). As AsA was applied on the leaf surface, where it first enters the apoplast, we further investigated whether the pro-oxidant activity was an apoplastic phenomenon or occurred also in the symplast. For this purpose, we applied AsA biosynthetic precursor, L-GalL that is not $Fe^{3+}$ reductant (data not shown), which can only be converted into AsA via the symplastic AsA biosynthesis (Alhagdow et al. 2007). Similar to direct AsA application, the precursor application increased the foliar AsA concentration and induced lipid peroxidation in the Fe treatment (Fig. 9). These results suggested that pro-oxidant activity of reduced AsA was not confined to the apoplast, but also occurred in the symplast. The association between ascorbate metabolism and response to Fe toxicity were further investigated in an independent pair of genotypes, which was selected from a global rice population previously screened in Fe toxic conditions (Matthus et al. 2015)(Fig. 8, 9; Supporting information Figure S4). Although these genotypes showed a similar trend (higher AO activity and lower DHAR activity in the tolerant genotype), the genotypic differences were less pronounced. Therefore, further investigations in larger and genetically diverse populations are warranted to confirm a general link between ascorbate turnover and leaf bronzing under Fe toxicity.

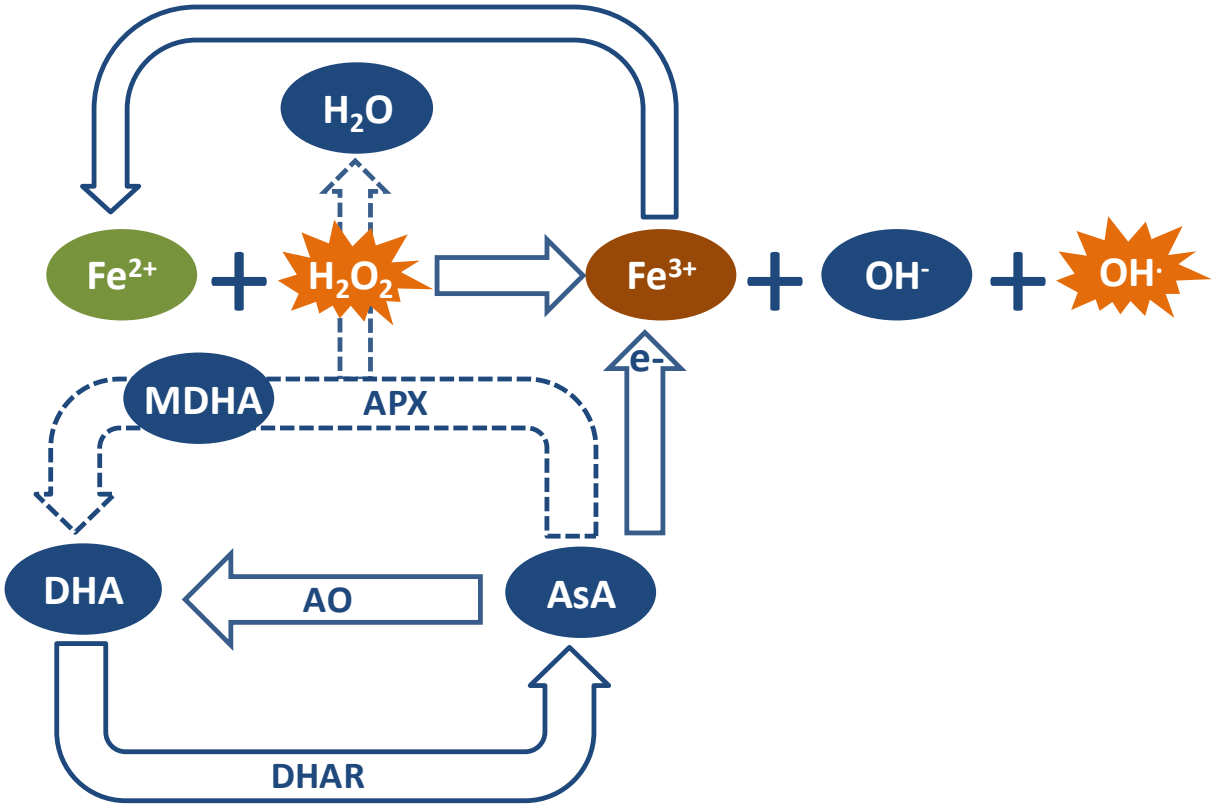


Figure 10. Proposed conceptual model of the pro-oxidant activity of ascorbate in the presence of $Fe^{2+}$ identified in IR29 and FL483. AsA, reduced ascorbic acid; AO, ascorbate oxidase; APX, ascorbate peroxidase; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; MDHA, mono-dehydroascorbic acid. The continuous recycling of AsA via DHAR is paralleled by reduction of $Fe^{3+}$ into $Fe^{2+}$, which stimulates the Fenton reaction to produce the hydroxyl radical. This process can be mitigated by ascorbate oxidase, which removes reduced ascorbate. In this concept the pro-oxidant processes dominates over the reduction of $H_2O_2$ into $H_2O$ via APX, as indicated by broken lines.

A conceptual model was developed in order to explain this hypothetical link (Fig. 10). Excess $Fe^{2+}$ ions react with $H_2O_2$ to generate hydroxyl radicals (the Fenton reaction), which are extremely reactive and cannot be scavenged by known plant antioxidants (Apel and Hirt 2004). Thus, plants could employ two strategies to avoid oxidative damage: (I) Keeping $H_2O_2$ under control through scavenging enzymes such as APX. However, we did not observe any genotypic significance in the expression or activity of enzymes involved in $H_2O_2$ reduction (Fig. 3, Supporting Information Fig. S2) although $H_2O_2$ levels indeed appeared to be lower in the tolerant genotype (Fig. 1). (II) A second strategy would be to prevent the reduction of $Fe^{3+}$ into $Fe^{2+}$. The redox potential of reduced AsA is sufficiently low to directly reduce Fe, as previously shown *in planta* in pea and *Arabidopsis* embryos acquiring Fe through the reduction of $Fe^{3+}$ ions by the efflux of ascorbate (Grillet et al. 2014). In the presence of excessive Fe,

this ability may stimulate the Fenton reaction. Therefore, a high rate of AsA turnover as represented by high DHAR activity in the sensitive IR29 leads to continuous formation of hydroxyl radicals via the Fenton reaction. In contrast, high AO activity, as seen in the tolerant FL483, removes reduced AsA from cells thereby slowing down the formation of $Fe^{2+}$ and consequently leads to less hydroxyl formation.

In conclusion, shoot-based tolerance to Fe toxicity in the two genotypes investigated in this study was associated with redox homeostasis of leaves as affected by the turnover of AsA. These results have implications for the breeding of crops tolerant to excess supply of redox active ions.

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# Chapter IV. Loci, genes and mechanisms associated with tolerance to ferrous iron toxicity in rice (*Oryza sativa* L.)

Matthus E., **Wu L.-B.**, Ueda Y., Höller S., Becker M. & Frei M. (2015) Loci, genes, and mechanisms associated with tolerance to ferrous iron toxicity in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, 1-14.

## 4.1 Abstract

Iron toxicity is a major nutrient disorder affecting rice. Therefore understanding genetic and physiological mechanisms associated with iron toxicity tolerance is crucial in adaptive breeding and biofortification. We conducted a genome-wide association study (GWAS) by exposing a population of 329 accessions representing all subgroups of rice to ferrous iron stress (1,000 ppm, 5 days). Expression patterns and sequence polymorphisms of candidate genes were investigated, and physiological hypotheses related to candidate loci were tested using a subset of contrasting haplotypes. Both iron including and excluding tolerant genotypes were observed, and shoot iron concentrations explained around 15.5 percent of the variation in foliar symptom formation. GWAS for seven traits yielded 20 SNP markers exceeding a significance threshold of $-\log_{10}P > 4.0$, which represented 18 distinct loci. One locus mapped for foliar symptom formation on chromosome 1 contained two putative glutathione-S-tranferases, which were strongly expressed under iron stress, and showed sequence polymorphisms in complete linkage disequilibrium with the most significant SNP. Contrasting haplotypes for this locus showed significant differences in dehydroascorbate reductase activity, which affected the plants' redox status under iron stress. We conclude that maintaining foliar redox homeostasis under iron stress represented an important tolerance mechanism associated with a locus identified through GWAS.

Keywords: Abiotic stress, biofortification, genome-wide association study, iron toxicity, oxidative stress, redox homeostasis, rice

## 4.2 Introduction

Iron (Fe) toxicity is one of the most commonly observed mineral disorders in rice production affecting millions of hectares of rice land, especially in Southeast Asia and West Africa (Audebert and Fofana 2009; Becker and Asch 2005). It specifically affects rice production, because the low soil redox potential of flooded rice paddies leads to the prevalence of the reduced and soluble $Fe^{2+}$ (ferrous), as opposed to well aerated soils, in which sparingly soluble forms of oxidized $Fe^{3+}$ (ferric) are dominant. Fe toxicity can occur on variable soil types, including acid sulfate or acid clay soils with inherently high Fe concentrations, or poorly-drained sandy soils in valleys receiving Fe-rich runoff water from adjacent slopes (Becker and Asch 2005). Fe toxicity causes substantial yield losses in rice and can lead to complete crop failure in severe cases.

Fe is an important plant nutrient and its enrichment in the grain is desirable from a human nutrition perspective (White and Broadley 2009), but foliar tissue concentrations above 300 ppm are considered as harmful for rice (Dobermann and Fairhurst 2000). Although rice plants possess sophisticated regulatory mechanisms of Fe uptake and homeostasis (Bashir et al. 2010), high amounts of $Fe^{2+}$ in the soil solution can lead to excessive $Fe^{2+}$ uptake, and its transport to the shoot via the transpiration stre am. Symptoms of Fe toxicity in rice become visible as necrotic brown spots on the leaves termed as ‘leaf bronzing’. These symptoms occur as a consequence of oxidative stress, as $Fe^{2+}$ catalyzes the generation of reactive oxygen species (ROS) via the ‘Fenton reaction’, in which hydrogen peroxide ($H_2O_2$) oxidizes $Fe^{2+}$ to produce hydroxide (OH-) and the highly reactive hydroxyl radical ($OH^{\cdot}$) (Becana et al. 1998). An imbalance of ROS generation in cells and their removal through antioxidants causes irreversible damage of different cellular components such as lipids, proteins, and DNA, and can induce cell death (Blokhina et al. 2003). The hydroxyl radical, which is produced in the presence of $Fe^{2+}$, is considered as the most toxic ROS for plant cells due to the lack of an effective scavenging mechanism through antioxidants (Apel and Hirt 2004).

The breeding of tolerant rice varieties constitutes a powerful approach to address the problem of Fe toxicity. In principle, two physiological strategies may be targeted in achieving this aim: (i) Exclusion of Fe at the root surface by oxidation of $Fe^{2+}$ into insoluble $Fe^{3+}$, which leads to the formation of a root plaque, *i.e*. precipitates of Fe at the root surface. Root architectural traits favoring this process include the formation of an aerenchyma and a large number of lateral fine roots, which facilitate the diffusion of oxygen into the rhizosphere, thereby increasing the redox potential above the threshold for Fe oxidation (Becker and Asch 2005; Wu et al. 2014). Alternatively, enzymatic Fe oxidation can be catalyzed by enzymes such as peroxidases (Becker

and Asch 2005). (ii) Various mechanisms have been proposed conferring 'shoot tolerance', *i.e*. the absence of stress symptoms despite high $Fe^{2+}$ uptake. Fe partitioning both on the organ and the subcellular level may constitute such a mechanism. For example storage of Fe in the leaf sheaths was proposed to be less damaging than in the photosynthetically more active leaf blades of rice (Engel et al. 2012). On the subcellular level, the vacuole constitutes an important compartment for the storage of excess metal ions (Moore et al. 2014). Much of the plants' excess Fe is stored in the form of ferritin, a ubiquitous protein occurring in almost all living species, which can store up to 4,000 atoms of Fe in a safe and bioavailable form (Arosio et al. 2009; Stein et al. 2009). Another mechanism of shoot tolerance could be the scavenging of ROS through the plants' antioxidant network, thus avoiding the formation of oxidative stress. However, plants do not possess effective scavengers of the hydroxyl radical – the product of the Fenton reaction (Apel and Hirt 2004). Therefore, antioxidants would have to remove the precursors of the hydroxyl radical such as hydrogen peroxide, which is reduced to water by antioxidant enzymes such as catalases and peroxidases (Blokhina et al. 2003).

The genetic architecture of tolerance to Fe toxicity in rice appears to be complex. Although quite a few studies reported quantitative trait loci (QTL) for different phenotypes related to Fe toxicity (Dufey et al. 2014; Wu et al. 2014), no major locus has been identified, fine-mapped, or cloned so far. Also, the tolerance rankings of genotypes tested under different natural and artificial environments have sometimes produced contradictory results (Wu et al. 2014), suggesting that the environmental conditions, the timing and level of Fe stress, the screening system, etc. play crucial roles in determining genotype responses to Fe toxicity. Despite these confounding factors, some convergence can be observed on several chromosomal regions, where independent studies reported QTL, including on chromosome 1 between around 25 to 30 Mb and on chromosome 3 between around 0 to 5 Mb (Dufey et al. 2015; Wu et al. 2014). One major limitation of these previous studies was that they all used bi-parental populations, thus covering only a small genetic variability not representing the enormous diversity of Asian rice (*Oryza sativa* L.). Also, the resolution of mapping in these previous studies was limited by a small number of genetic markers (several hundred) and the typically limited number of chromosomal recombination events occurring in bi-parental crosses (Huang and Han 2014).

To overcome these limitations, we aimed at unravelling genetic and physiological mechanisms underlying tolerance to Fe toxicity by screening a highly diverse population of 329 rice accessions representing the global genetic diversity of rice (Zhao et al. 2011). Screening experiments were followed by a genome-wide association study (GWAS) employing 44,100 single nucleotide polymorphism (SNP) markers to determine candidate loci linked to tolerance. Lastly, one candidate locus,

which co-localized with previously reported QTL, was investigated in further detail by sequence and expression analyses of candidate genes, and tests of hypotheses related to the physiological mechanism underlying the locus.

## 4.3 Materials and methods

### *4.3.1 Phenotyping experiment*

The population used for genome-wide association study was composed of 329 Asian rice (*Oryza sativa* L.) varieties, originating from 77 countries, covering all major rice-growing regions and five subpopulations of rice, including 74 *indica,* 55 *aus,* 69 *temperate japonica,* 70 *tropical japonica* and 12 *aromatic* varieties. Additional 49 accessions were classified as mixed, showing less than 80% ancestry from any single sub-group. Genotypic data for 44,100 SNPs was publicly available for each line (Zhao et al. 2011). Seeds were obtained from the International Rice Research Institute (IRRI).

Screening experiments were conducted in a hydroponic system (Engel et al. 2012) in the greenhouses of the University of Bonn, Germany, with a 12 h photoperiod, 650 µmol $m^{-2}$ $s^{-1}$ illumination (PAR), and 30 °C/25 °C day/night temperature. Twenty five seeds per line were germinated in distilled water, and then transferred to netted styrofoam trays floating on 0.5 mM $CaCl_2$ and 10 µM $FeCl_3$ solution. After two weeks, homogenous seedlings of each line were transplanted to 60 L hydroponic containers filled with a modified Yoshida Solution (Yoshida et al. 1976), which had the following full strength composition: N 2.86 mM (as $NH_4NO_3$), P 0.26 mM (as $NaH_2PO_4x2H_2O$), K 0.82 mM (as $K_2SO_4$), Ca 0.8 mM (as $CaCl_2$), Mg 1.3 mM (as $MgSO_4x7H_2O$), Mn 7.3 µM (as $MnCl_2x4H_2O$), Mo 0.4 µM (as $(NH_4)_6Mo_7O_{24}x4H_2O$)), B 14.8 µM (as $H_3BO_3$), Zn 0.16 µM (as $ZnSO_4x7H_2O$), Cu 0.16 µM (as $CuSO_4x5H_2O$), Fe 28.7 µM (as $FeCl_3x6H_2O$). Plants were fixed with sponges on a perforated lid, and the root of each single plant was separated by a PVC- tube fixed underneath the lid to allow each genotype to form its own rhizosphere. To avoid osmotic stress of young seedling, plants were grown in half strength nutrient solution for the first ten days. The pH was adjusted to 5.5 twice a week and the solutions were renewed every ten days.

Until the start of Fe-stress, all containers were arranged randomly in the greenhouse to minimize the impact of spatial differences in microclimate. Each 60 L container contained 40 different and randomly arranged lines. Each experimental cycle included four plants of each genotype in both experimental conditions, of which two were grown in the same planting hole, respectively. This added up to 17 containers for control conditions and 17 containers for the Fe-stress treatment. Due to space and labor constraints, but to obtain eight replicate plants per treatment and genotype, two cycles of the experiment described above were carried out from March until June 2013. In summary, eight replicate plants of each line were screened under control conditions and eight replicate plants under Fe stress conditions.

A 5-day Fe pulse stress of 1,000 ppm $Fe^{2+}$ (as $FeSO_4x7H_2O$) was imposed four weeks after the transplanting. This treatment aimed at simulating acute Fe toxicity during the vegetative growth stage, as it typically occurs in inland valleys during intensive rainfall events (Becker and Asch 2005). To avoid oxidation and precipitation of $Fe^{2+}$, the solutions were automatically percolated with $N_2$ gas for 15 min every 2 h. This kept the redox potential below the threshold for iron oxidation (Engel et al. 2012).

As a measure of Fe-stress, a leaf bronzing score (LBS) was assigned to the three youngest fully expanded leaves of the main tiller of each plant on day three (LBS3) and day five (LBS5) after the application of the stress treatment. The score ranged from 0 (healthy leaf) to 10 (dead leaf) as described by Wu et al. (2014). After five days of treatment, all plants were harvested, and the number of tillers, shoot height and root length of each plant were determined. The samples were dried at 70 °C until completely dry and weighed. For Fe concentration analyses, the eight shoot samples from the Fe treatment of each line were pooled and finely ground. A representative sample was analyzed in duplicate by atomic absorption spectrometry as previously described (Wu et al. 2014).

*4.3.2 Hypothesis testing experiment*

A second experiment was conducted during August/September 2014 to test physiological hypotheses using a subset of lines representing different haplotypes for specific loci. The experimental conditions and experimental systems were identical to those described above. Six hydroponic containers were assigned to three replicate tanks for control and Fe stress treatment, respectively. Each tank contained four replicate plants of each of the eight genotypes tested (single plants per hole). Plant samples were harvested around midday, immediately frozen in liquid nitrogen and stored at -80 °C for further analysis.

*4.3.3 Data analysis and association mapping*

Two-way ANOVA tests were applied to analyze effects of treatment, genotype, and the interaction of both on the phenotypic traits. In both cases, PROC GLM in SAS 9.3 (SAS Institute Inc. Cary, NC) was used and Tukey's HSD test was applied to separate means.

A mean value of the eight replicates of the phenotypic values was used for association mapping. To remove extreme values in each trait, data points which did not fall into the range of (mean of all accessions) ± (3 times the standard deviation) were removed from the dataset prior to the mapping, which led to the exclusion of a maximum of 5 values in the case of Fe concentration. To ensure normal distribution of data, square-root transformation was conducted for LBS prior to the mapping, as

this trait showed skewed distribution in the original dataset. One-way ANOVA was conducted for subpopulation comparison followed by Tukey's HSD for the post-hoc test.

Association mapping was conducted for the whole population and for the subgroups (*japonica* and *indica*) separately, using the software TASSEL 3.0 (Bradbury et al. 2007). SNP marker data, kinship matrix and principal component analysis (PCA) matrix retaining the four main axes were described previously (Zhao et al. 2011). This SNP array provides approximately one SNP marker every 10 kb. SNPs which showed minor allele frequency (MAF) <5% in our population were removed to avoid overestimation of the effect of SNPs with low frequency. The resultant number of SNPs was 34 564 for the whole panel and 26,621 and 17,106 for the subgroups *indica* and *japonica*, respectively. A mixed linear model (MLM) was used to calculate associations in all analyses, incorporating both PCA and kinship data. The MLM was applied using the default settings (P3D for variance component analysis, compression level set to optimum level). A significance threshold was set to $-\log_{10}P > 4.0$ for in-depth analysis of the most significantly associated markers such as linkage block analysis and curation of candidate gene lists. This threshold was justified by a steep decrease of corresponding q-values (Storey and Tibshirani 2003) in the $-\log_{10}P > 4.0$ region, and was also applied by other authors using the same population (*e.g*. Wissuwa et al. 2015). In addition to this significance threshold approach, the 50 most strongly associated markers for each trait were curated as proposed by Verslues et al. (2014) to analyze co-localization of strongly associated markers for different traits, even though they may not exceed a significance value of $-\log_{10}P > 4.0$. Linkage disequilibrium (LD) blocks were defined by the confidence interval method (Gabriel et al. 2002) with default settings using Haploview 4 program (Barrett et al. 2005) based on D' values deriving from the SNPs with MAF > 5%. LD blocks harboring significant SNPs were then defined as the candidate loci. The annotations of genes located in these loci were obtained from the MSU7 rice genome database (http://rice.plantbiology.msu.edu/, as of October 2014). LD between significant markers and putative functional polymorphisms within candidate genes were assessed by $r^2$ (Chen et al. 2014) as it takes into account the history of both recombination and mutations (Flint-Garcia et al. 2003).

*4.3.4 RNA extraction and quantitative RT-PCR*

Shoot samples from the hypothesis testing experiment were immediately frozen in liquid $N_2$ and stored at -80 °C. For quantitative real time polymerase chain reaction (qPCR), mRNA was extracted from three samples per haplotype and treatment using the peqGOLD Plant RNA Kit (Peqlab, Erlangen, Germany). DNA was removed from RNA samples by on-column digestion using a peqGOLD DNase I Digest Kit (Peqlab).

RNA concentration was determined using a Nanodrop2000C spectrometer (Thermo Scientific) and the integrity of RNA was checked by denaturing formaldehyde agarose gel electrophoresis. Three hundred ng of total RNA were reverse transcribed with the GoScript™ Reverse Transcription System (Promega, Mannheim, Germany) and qPCR was performed using the GoTaq® qPCR master mix (Promega) using a StepOne Plus Realtime PCR System (AB Biosystems, Darmstadt, Germany), and the following conditions (Höller et al. 2014): an initial denaturation step (10 min, 95 °C), followed by 40 cycles of denaturation (15 sec, 95 °C) and annealing/extension (1 min, 60 °C). Gene specific primers were as follows: LOC_Os01g49710 forward 5'-CCTGGAGCACTACAAGGGAT-3', reverse 5'-CGAGCAAGGCAGATAGATTG-3'; LOC_Os01g49720 forward 5'-CGTCGTCCTGGAGTACATC-3', reverse 5'-GACCTGAACAGCACTTTCC-3'. Expression data were quantified using the comparative $\Delta\Delta C_T$ method with the expression level of the sensitive line Guan-Yin-Tsan in the control treatment as calibrator and 18S rRNA as endogenous reference (Frei et al. 2010). Primer efficiency was tested through serial dilutions of cDNA templates and was always above 80%.

*4.3.5 DNA extraction and sequence analyses*

Genomic DNA from selected lines was extracted from plant shoots using a PeqGold plant DNA extraction kit (Peqlab). The region of interest was amplified by PCR with the following setup: 25 µL of GoTaq green master mix, 1 µL of each primer (10 µM), 2.5 µL of dimethyl sulfoxide, 15.5 µL of water and 70 ng of template DNA. The following conditions were used for amplification: 95 °C for 2 min, 35 cycles of 95 °C for 30 sec, 57/55 °C for 30 sec, and 72 °C for 2/1.5 min, followed by an additional 72 °C extension for 5 min. The primer sequences were 5'-CTTTCTGTGATTTGCGATGT-3' / 5'-CCGATTCCAACTTTGCTTA-3' for LOC_Os01g49710 and 5'-GGTGTCTGTAACTACTTCCAGTC-3' / 5'-AACTTATTCAACCTGCAATCCCTC-3' for LOC_Os01g49720. The amplified DNA was purified after gel electrophoresis using a kit (FastGene Gel/PCR Extraction Kits, Nippon Genetics, Tokyo, Japan). The purified DNA was subjected to a cycle sequencing using the primers used for the PCR. Due to the sequence reading limit, a third primer 5'-GTTCAAGGTGGTGGACGAG-3' was used to complete the sequencing of LOC_Os01g49710. Sequences were compared and analyzed using MEGA 5 software (Tamura et al. 2011). Additional genomic sequences were obtained from the TASUKE rice genome browser (http://rice50.dna.affrc.go.jp/) (Kumagai et al. 2013).

### 4.3.6 Biochemical analyses

Reduced and oxidized ascorbic acid (AsA) was measured in shoots immediately after harvesting (Ueda et al. 2013). Shoot and leaf material was ground in liquid nitrogen

and about 80 mg of sample material were dissolved in 1 ml 6 % metaphosphoric acid (MPA) containing 1 mM ethylene-diamine-tetraacetic acid (EDTA). Samples were centrifuged at 15,000 g and 4 °C for 20 min. Supernatants were used for further analyzes. For the measurements of reduced AsA, 10 µl of the extracts were added to the reaction mix containing 100 mM potassium phosphate buffer (pH 7.0) and 0.1 units of ascorbate oxidase (AO). In the case of oxidized AsA, 10 µl of the extract were added to 100 mM potassium phosphate buffer (pH 7.8) and 4 mM dithio-threitol (DTT). Absorbance was monitored in a microplate reader (Powerwave XSII, BioTek, Bad Reichenhall, Germany) at 265 nm in UV-transparent 96-well microplates until it was constant ($\varepsilon$ =14.3 $mM^{-1}$ $cm^{-1}$). Total AsA was calculated as the sum of reduced and oxidized AsA.

Glutathione concentration was determined spectrometrically (Griffith 1980). Plant shoots were crushed in liquid N and glutathione was extracted from around 100 mg using 1.5 ml of 4% sulfosalicylic acid and 5% insoluble polyvinyl-polypyrrolidone (PVPP). Samples were centrifuged at 9,400 g for 10 min at 4 °C, and the supernatant was neutralized by adding 1 M $KH_2PO_4$/$K_2HPO_4$. The reaction mix (100 µl) contained 0.6 mM 5,5-dithiobs-(2- nitrobenzoic acid) (DTNB), 0.2 mM NADPH, and 20 µl of the extract. The reaction was started by adding 0.125 units of glutathione reductase and followed at 412 nm for 3 min. Oxidized glutathione (GSSG) was determined by irreversible derivatization of reduced glutathione (GSH) with 2-vinylpyridine prior to the DTNB reaction.

Enzyme activities were monitored in a microplate reader using 96-well microplates. The activities of ascorbate peroxidase (APX), monodehydroascorbate reductase(MDHAR) and glutathione reductase (GR) were measured using the same plant extract (Frei et al. 2012). Around 100 mg of flash-frozen and ground leaf material were dissolved in 1 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 10,000 g and 4 °C. The reaction mix for APX activity (100 µl) contained 100 mM potassium phosphate buffer (pH 6.8), 0.6 mM AsA, 10 µl of 0.03 % $H_2O_2$, and 10 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 min ($\varepsilon$ = 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 GSSG and 10 µl of plant extract. Oxidation of NADPH was monitored at 340 nm for 3 min ($\varepsilon$ = 6.2 $mM^{-1}$ $cm^{-1}$).

For determination of dehydroascorbate reductase (DHAR) activity (Hossain and Asada 1984), approximately 100 mg of flash-frozen leaf material were 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 13,000 g for 5 min at 4 °C. The reaction mix (100 µl) contained 50 mM potassium phosphate buffer (pH 6.5), 0.5 mM DHA, 5 mM GSH and 10 µl of plant extract. Absorbance was followed at 265 nm for 3 min ($\varepsilon$ = 14 $mM^{-1}$ $cm^{-1}$). Protein concentrations in enzyme extracts were determined according to Bradford (1976).

## 4.4 Results

### *4.4.1 Phenotypic response*

Seven phenotypic traits were measured, including biomass and growth-related traits, symptom formation and Fe concentrations. As we applied an acute stress for a short period of time (five days) to around five-weeks old plants, biomass-related traits were not strongly affected. On average of all accessions, shoot dry weight was not significantly reduced and root dry weight even increased by around 30% ($P$ <0.001). Average root length was not affected by the Fe treatment and shoot length slightly decreased by around 4% ($P$ <0.05). Stress symptoms, as represented by leaf bronzing score (LBS), began to develop after three days of Fe treatment, and were clearly visible in most accessions five days after treatment application. Fe concentrations were measured only in the Fe treated shoots, since our previous experiments (Engel et al. 2012; Wu et al. 2014) had shown that plants grown under Fe toxicity had shoot Fe concentrations several orders of magnitude higher than in control treatments, and therefore do not correlate with values observed under control conditions. Symptom formation (LBS5) and shoot Fe concentration were positively correlated ($-\log_{10}P$ > 4.0, Fig.1A), but the $r^2$ value indicated that Fe concentrations explained only 15.5% of the observed variation in leaf bronzing scores. When removing the outlier value with an Fe concentration of 9.2 mg $g^{-1}$ dry weight (Fig.1A), the $r^2$ value was even lower (0.1389) but the correlation was still significant ($-\log_{10}P$ >4.0). Tolerant includers, *i.e*. lines with low symptom formation despite high shoot Fe concentration, were represented in all subpopulations of rice (Fig.1A), indicating that this trait was not specific to any subpopulation. We further compared subpopulation differences for those traits, which we focused on in the association mapping. It was found that *aromatic* and temperate *japonica*s had significantly lower LBS5 and shoot Fe concentrations, suggesting that Fe exclusion was a dominant tolerance mechanism in these subpopulations (Fig.1B, C). Total Fe uptake was less correlated with symptom formation than Fe concentration and explained less than five percent of the phenotypic variation (Supplementary Figure S1).

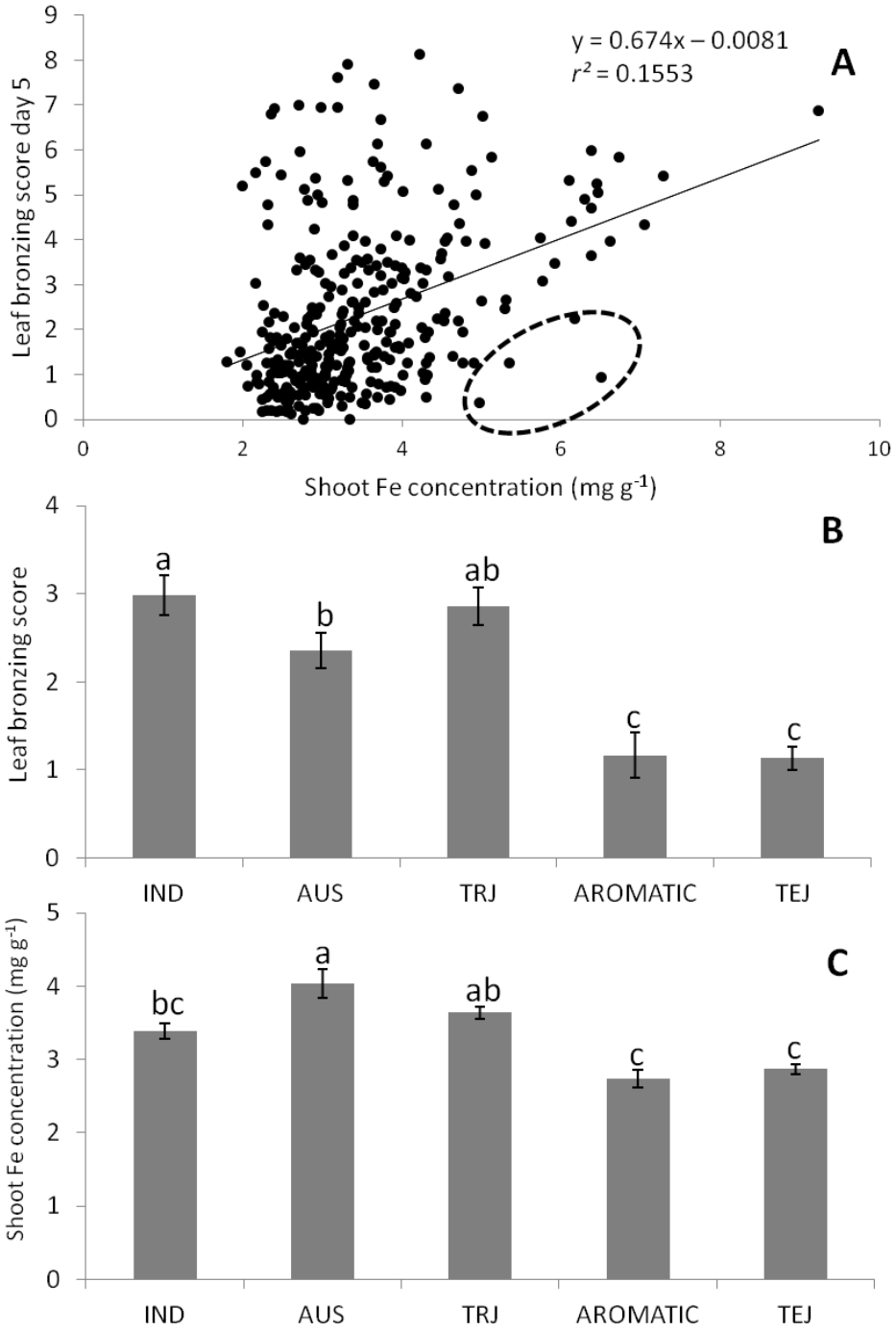


Figure 1. Analysis of leaf bronzing scores and shoot Fe concentrations in 329 rice lines exposed to Fe toxicity (1,000 ppm) for five days. (A) Linear regression of shoot Fe concentrations versus leaf bronzing scores. Encircled data points represent tolerant includers, *i.e.* accessions with little symptom formation despite high Fe concentration: DM59 (*aus*), Jaya (*indica*), Kamenoo (temperate *japonica*) and Khao Hawm (tropical *japonica*) (B) Sub-population analysis of leaf bronzing score; (C) Sub-population analysis of shoot Fe concentrations; IND indica, TRJ tropical japonica, TEJ temperate japonica. Data bars show mean values and standard errors; bars not sharing the same letter are significantly different at $P$ <0.05 by Tukey's HSD test.

*4.4.2 Association mapping*

Association mapping for all traits included the determination of SNPs exceeding a significance value of $-\log_{10}P > 4.0$, curation of the top 50 most significant SNPs, analyses of LD blocks surrounding each highly significant ($-\log_{10}P > 4.0$) SNPs, and curation of a list of candidate genes contained within these blocks (Supplementary Figures S2-S6, Supplementary Data S7). However, detailed analyses are presented only for those traits which we considered as the most relevant under our experimental conditions, *i.e*. LBS5 and shoot Fe concentration. Square root transformed leaf bronzing score (tLBS5) showed nearly normal distribution (Fig.2A). Mixed model analysis yielded quantile-quantile-plots (q-q-plots), which showed upward deviation from the expected $-\log_{10}$(*P*-values) only for the most significantly associated markers (Fig.2B). The significance threshold of $-\log_{10}P > 4.0$ for the association of SNPs with tLBS5 was exceeded in two chromosomal regions (Fig.2C). On chromosome 1, three highly significant markers were located in two nearby LD blocks (Fig.2D), which were co-localized with several previously reported QTL (Dufey et al. 2014; Wu et al. 2014). The first block spanned around 82 kb and contained two highly significant SNPs ($-\log_{10}P > 4.0$), and 15 gene models (MSU7), including an FAD-binding and arabino-lactone oxidase domains containing protein (LOC_Os01g49360). The second block spanned around 50 kb and contained 14 gene models. The most significant SNP marker (id1016768) was located close to two putative glutathione S-transferase genes (LOC_Os01g49710, LOC_Os01g49720). We further analyzed phenotypic means of different haplotypes for the three most significant markers on chromosome 1 (Fig. 2E). The most sensitive haplotype TGC only occurred in the *indica* subgroup and was significantly more sensitive than the haplotypes CTA and TGA. These analyses also showed that the A/C SNP id1016768 had the largest effect on the phenotypic means, as the C allele occurred only in the most sensitive haplotype TGC.

A second region on chromosome 5 contained one further SNP exceeding $-\log_{10}P > 4.0$ (id5000074) which was located in a 174 kb LD block. This region did not co-localize with any previously reported QTL (Dufey et al. 2014; Wu et al. 2014). Out of the 36 gene models contained in this linkage block possible candidate genes related to Fe tolerance included a putative vacuolar ATP-synthase (LOC_Os05g01560).

Association mapping for shoot Fe concentration yielded somewhat lower significance values (Fig.3) with only two SNPs exceeding $-\log_{10}P > 4.0$. The most significant SNP (id2015632) was located within a 12 kb LD block on chromosome 2 (Fig.3D). This block contained only three gene models, one of which was annotated as a casein kinase I (LOC_Os02g56560). The locus was localized near previously reported QTL for leaf bronzing score and photosynthesis under Fe toxic conditions reported earlier

(Dufey et al. 2014; Wu et al. 2014). The second highly significant marker (id1015380) was located outside any LD block on chromosome 1, but was localized within the broader region (25-30 Mb) on chromosome 1, in which several previously reported QTL are co-localized (Dufey et al. 2014; Wu et al. 2014). Gene models located near the marker and between the two adjacent linkage blocks included a putative potassium ion channel (LOC_Os01g45990).

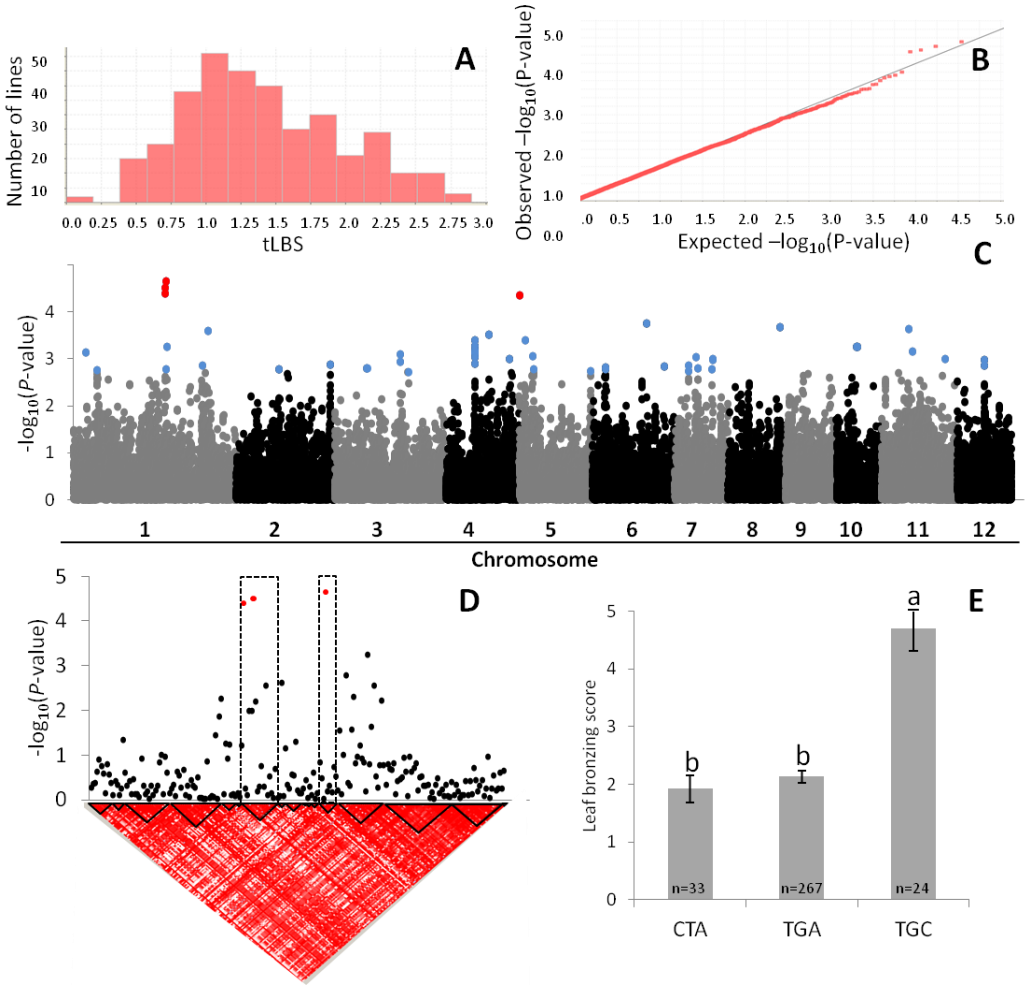


Figure 2. Association mapping results for square-root-transformed leaf bronzing score (tLBS5) after five days of Fe treatment. (A) Frequency distribution of tLBS5 in the association panel. (B) q-q-plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying P-values for each marker according to mixed linear model analysis. Blue dots indicate the top 50 SNPs ($-\log_{10}P$ values ranging from 2.72-4.64) and red dots indicate SNP exceeding a significance level of $-\log_{10}P > 4.0$. (D) Linkage block analysis of the candidate locus on chromosome 1; a 1.45 Mb region spanning from 27.85 to 29.30 Mb is shown; triangles framed in black indicate linkage blocks determined as stated in the materials and methods section. (E) Average leaf bronzing score of contrasting haplotypes for the highly significant ($-\log_{10}P$ >4.0) markers (id1016591, id1016614, and id1016768) on chromosome 1. Data bars show mean values and standard errors; bars not sharing the same letter are significantly different at $P$ <0.05 by Tukeys HSD test.

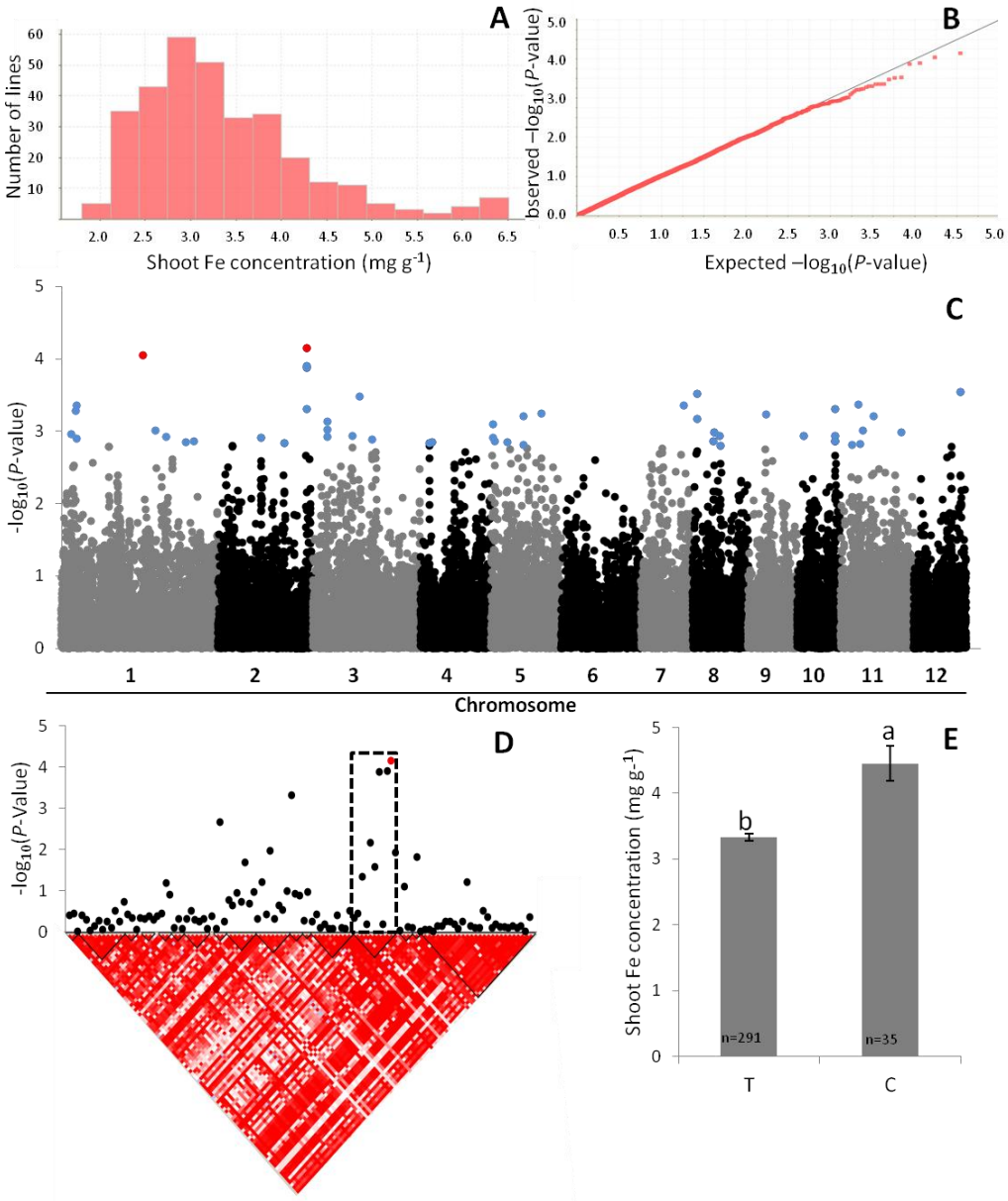


Figure 3. Association mapping results for shoot Fe concentration after five days of Fe treatment. (A) Frequency distribution of Fe concentrations in the association panel. (B) q-q-plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying P-values for each marker according to mixed linear model analysis. Blue dots indicate the top 50 SNPs ($-\log_{10}P$ values ranging from 2.80-4.15) and red dots indicate SNP exceeding the significance level of $-\log_{10}P > 4.0$. (D) Linkage block analysis of the candidate locus on chromosome 2; a 0.63 Mb region spanning from 34.21 to 34.84 Mb is shown; triangles framed in black indicate linkage blocks determined as stated in the materials and methods section. (E) Average Fe concentrations of lines representing different alleles at the highly significantly ($-\log_{10}P > 4.0$) associated SNP marker (id2015632). Data bars show mean values and standard errors; bars not sharing the same letter are significantly different at $P < 0.05$ by t-Test.

*4.4.3 Analysis of candidate locus for tLBS5 on Chromosome 1*

Among the candidate loci identified through association mapping, we opted to analyze in more detail the locus associated with tLBS5 on chromosome 1 (Fig.2C, D, E) as this peak was consistently seen with different mapping approaches: (i) the peak occurred when using a general linear model (GLM, data not shown), as well as the MLM analysis presented here; (ii) the peak also occurred when the MLM was applied to the *indica* subgroup alone (Supplementary Data S7); (iii) for the *indica* subgroup the most significant markers of this peak (Fig.2) were also among the top 50 SNPs for tLBS3 (Supplementary Data S7), and (iv) several previous studies with different bi-parental populations had reported QTL for Fe toxicity tolerance in this chromosomal region (Dufey et al. 2014). Eight contrasting *indica* lines were selected to test the hypotheses that (i) Fe toxicity tolerance is related to differential expression or sequence polymorphisms of the candidate genes located in this locus: the glutathione-S-transferase (GST) genes LOC_Os01g49710 and LOC_Os01g49720, and (ii) the locus is associated with differential antioxidant activity. Genomic sequences were analyzed in 30 representative lines. Antioxidants were measured in a sub-set of eight selected lines, four of which represented the sensitive haplotype TGC for the three SNP markers exceeding the significance value of $-\log_{10}P > 4.0$ (Fig.2), and another four lines represented the tolerant haplotype CTA. These eight lines did not differ significantly in shoot Fe concentration, which was 3.56 mg $kg^{-1}$ on average for the sensitive haplotypes and 3.23 mg $kg^{-1}$ for the tolerant haplotypes, confirming that differences in tolerance occurred due to a shoot-based mechanism rather than Fe exclusion.

Alignment of the genomic sequences of 30 selected lines revealed seven polymorphisms in the gene model LOC_Os01g49710, nine polymorphisms in LOC_Os01g49720, and two polymorphisms upstream of the 5'UTR of LOC_Os01g49720 (Fig.4A). Five of these polymorphisms were in complete LD ($r^2$=1) with the nearby marker id1016768, which had the most significant *P*-value for tLBS5. Among those polymorphisms, one SNP was located upstream of the 5'UTR of LOC_Os01g49720, and one SNP was located in the coding sequence of LOC_Os01g49720. These SNP did not change the predicted amino acid sequence of the protein or cause any genotypic difference in mRNA expression (Fig. 4C). In contrast, three insertion-deletions (INDELs) in LOC_Os01g49710 were in complete LD ($r^2$=1) with the marker id1016768 (Fig.4A). The sensitive haplotype TGC had an 18-nucleotide insertion (GST1_INDEL50) in the first exon, which added six glycine units to the predicted protein sequence, and another 2-nucleotide frameshift deletion (GST1_INDEL734) in the second exon. A third 1-nucleotide insertion was located in the 3'UTR (GST1_INDEL978). Both candidate genes showed highly significant mRNA up-regulation under Fe stress (Fig. 4B, C), but no significant difference

between the haplotypes. In summary, these data demonstrated that both candidate genes were highly responsive to Fe-stress, and had sequence polymorphisms in complete LD with the most significant SNP marker, which in the case of LOC_Os01g49710 may cause substantial modifications in the protein structure.

Based on the detected sequence polymorphisms in GSTs, we hypothesized that contrasting haplotypes would differ in antioxidant response, and more specifically in dehydroascorbate reductase (DHAR) activity, which had previously been described as one possible function of GSTs (Edwards and Dixon 2005; Marrs 1996). Contrasting haplotypes did not differ significantly in foliar AsA concentration, but a significant treatment by haplotype interaction was observed in AsA redox state (Table 1): while the tolerant haplotypes had a more oxidized AsA pool under control conditions, the opposite was seen under Fe toxicity. Significant haplotype differences in AsA redox state were consistent with differential DHAR activity, which was significantly up-regulated under Fe-stress and significantly lower in the tolerant haplotypes. Total glutathione concentration, MDHAR activity, and glutathione reductase activity were significantly up-regulated under Fe-stress, but no significant haplotype effects were detected. In summary, our analyses demonstrated that differences between lines representing the tolerant and sensitive haplotype occurred mostly in DHAR activity and AsA redox state.

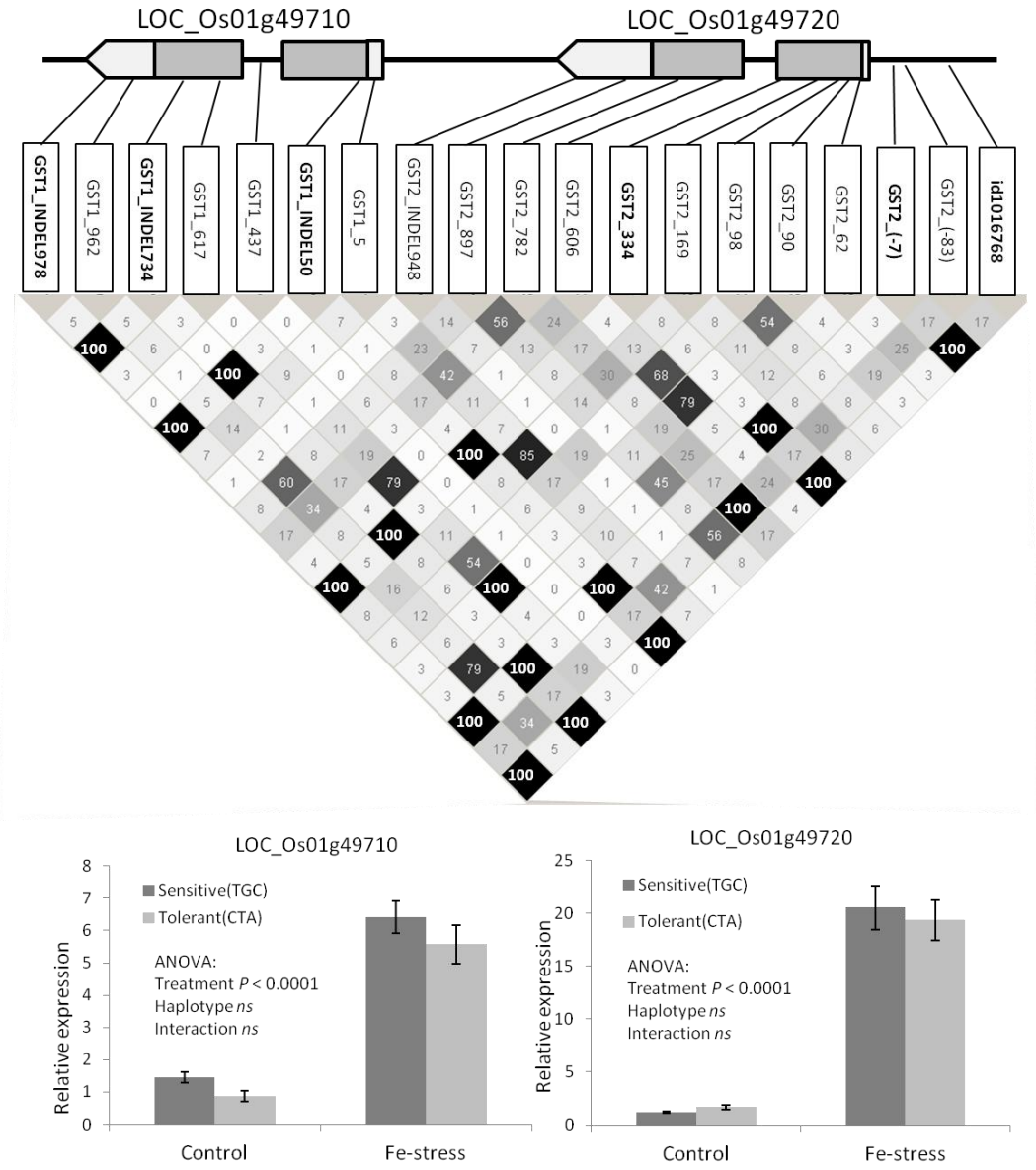


Figure 4. Sequence and expression analyses of candidate genes LOC_Os01g49710 and LOC_Os01g49720 putatively encoding glutathione-S-transferases. a Genomic sequences of 30 representative accessions were aligned. The polymorphisms shown in framed *vertical boxes* represent SNPs unless the acronym includes 'INDEL', in which casethey represent an insertion or deletion. The number following theacronym represents the position of the polymorphisms in the MSU7Nipponbare reference genome counting from the transcript initiation site. The triangular matrix indicates linkage disequilibrium ($r^2$ × 100) between polymorphic sites and the nearby SNP marker id1016768, which had the most significant *P* value for tLBS5. (b, c) Quantitative mRNA expression of LOC_Os01g49710 and LOC_Os01g49720in contrasting haplotypes under control and Fe-toxic conditions. Haplotypeswere defined as in Fig. 2e. Three representative lines per haplotypewere analyzed with three experimental replicates in both treatments (n = 36).

**Table 1.** Substrates and enzymes of the ascorbate-glutathione cycle in contrasting haplotypes differing in leaf bronzing symptoms after five days of exposure to Fe toxicity.

| **Variable** | **Control** | | **Fe-toxicity** | | **ANOVA Pr > F** | | |
|---|---|---|---|---|---|---|---|
| Haplotype | Tolerant (CTA) | Sensitive (TGC) | Tolerant (CTA) | Sensitive (TGC) | Treatment | Haplotype | Interaction |
| Total AsA (µmol $g^{-1}$ FW) | 2.75 | 2.58 | 2.54 | 2.67 | 0.5595 | 0.8272 | 0.1687 |
| Reduced AsA (µmol $g^{-1}$ FW) | 1.90 | 2.10 | 1.95 | 1.92 | 0.6757 | 0.5981 | 0.4437 |
| DHA (µmol $g^{-1}$ FW) | 0.85 | 0.48 | 0.58 | 0.75 | 0.9934 | 0.3835 | 0.0279 |
| AsA redox state (%) | 68 | 81 | 78 | 72 | 0.8806 | 0.3616 | 0.0439 |
| Total glutathione (µmol $g^{-1}$ FW) | 0.40 | 0.35 | 0.54 | 0.42 | 0.0334 | 0.1007 | 0.5415 |
| GSH (µmol $g^{-1}$ FW) | 0.33 | 0.29 | 0.45 | 0.36 | 0.0560 | 0.1891 | 0.6536 |
| GSSG (µmol $g^{-1}$ FW) | 0.06 | 0.06 | 0.08 | 0.06 | 0.2444 | 0.0688 | 0.3701 |
| Glutathione redox state (%) | 80 | 81 | 82 | 84 | 0.4844 | 0.6919 | 0.8207 |
| APX activity (µmol $mg^{-1}$ protein $min^{-1}$) | 1.04 | 1.32 | 1.42 | 1.99 | 0.0768 | 0.1478 | 0.6106 |
| DHAR activity (µmol $mg^{-1}$ protein $min^{-1}$) | 0.12 | 0.20 | 0.24 | 0.29 | 0.0002 | 0.0080 | 0.5426 |
| MDHAR activity (nmol $mg^{-1}$ protein $min^{-1}$) | 144 | 138 | 101 | 99 | 0.0017 | 0.7178 | 0.8368 |
| GR activity (nmol $mg^{-1}$ protein $min^{-1}$) | 26 | 28 | 38 | 40 | <0.0001 | 0.0549 | 0.8871 |

Redox state refers to the percentage of reduced AsA or glutathione. Haplotypes refer to SNPs on chromosome 1 exceeding the significance threshold of −log10 $P > 4.0$ for tLBS5 (id1016591, id1016614, and id1016768). Each haplotype was represented by four accessions: CTA Tchibanga (Gabon), Taichung Native 1 (Taiwan), ZHE 733 (China), Sadu Cho (Korea); TGC Guan-Yin-Tsan (China), Kiang-Chou-Chiu (Taiwan), Ming-Hui (China), RTS4 (Vietnam). Three biological replicates were analyzed per accession and LS means of 12 samples are shown (n = 48) *AsA* ascorbic acid, *DHA* dehydroascorbic acid, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *APX* ascorbate peroxidase, *DHAR* dehydroascorbate reductase, *MDHAR* monodehydroascorbate reductase, *GR* glutathione reductase

## 4.5 Discussion

With 329 different rice accessions from all subpopulations of *O. sativa*, our study represents an extensive screening for tolerance to Fe toxicity in rice, and demonstrated considerable variability in tolerance. We considered leaf symptom formation as a relevant phenotype related to Fe tolerance, similar to a number of screening and bi-parental QTL mapping studies reported previously (Dufey et al. 2009; Dufey et al. 2012; Dufey et al. 2014; Wu et al. 2014), as LBS was demonstrated to be highly correlated with yield formation under Fe-toxic field conditions (Audebert and Fofana 2009). Tolerance rankings reported in different studies sometimes produced contradictory results, presumably due to differences in environmental conditions (e.g. vapor pressure deficit, growth medium), as well as the timing and the intensity of Fe-stress (Wu et al. 2014). The tolerance ranking and associated loci presented in this study refer to an acute Fe-stress during the vegetative growth stage, as it typically occurs in inland valleys receiving Fe-rich runoff water from adjacent slopes (Becker and Asch 2005). Which tolerance mechanism was dominant under these circumstances: Fe exclusion at the root surface, or rather shoot-based mechanisms conferring tolerance despite high Fe uptake? Our results suggest that both mechanisms play a role: the correlation between shoot Fe concentration and LBS was significantly positive ($-\log_{10}P > 4.0$, Fig. 1A), suggesting that high Fe uptake was indeed a factor contributing to the formation of visible stress symptoms. However, the linear regression model explained only 15.5% of the phenotypic variation in LBS, leaving 84.5% of the variation unexplained by foliar Fe concentrations. That both tolerance mechanisms play a role under acute Fe stress in the vegetative stage is in agreement with our previous study (Wu et al. 2014), which identified QTL associated with both exclusion and inclusion mechanisms in bi-parental populations.

In the association mapping, we followed an approach based on the analysis of LD blocks surrounding significant SNPs, as previously adopted in an association study on tropospheric ozone stress (Ueda et al. 2015). The resolution of association mapping typically depends on the LD: fast LD decay leads to high resolution of mapping but requires a large number of markers to take advantage of many recombination events (Han and Huang 2013; Huang and Han 2014). For the traits tLBS5 and shoot Fe concentration, which are discussed in detail, the identified candidate regions were characterized by a varying degree of LD decay leading to candidate loci (LD blocks) ranging between 12 kb and 173 kb, containing between 4 to 36 gene models. In our previous GWAS dealing with ozone tolerance, even higher variability in the size of candidate regions was observed, ranging from less than 1 kb to more than 1 Mb. Thus, we assume that using a fixed window approach for declaration of candidate loci (Guo et al. 2009) would be more susceptible to declaring

false positive or false negative candidate genes than the approach based on LD blocks.

The analysis of candidate genes was based on annotated genes, although we cannot exclude the possibility of a non-annotated gene being responsible for the phenotype. The candidate LD blocks surrounding highly significant ($-\log_{10}P > 4.0$) SNPs contained various genes with possible involvement in Fe-homeostasis based on their MSU7 annotations (Kawahara et al. 2013). A putative vacuolar ATP-synthase (LOC_Os05g01560) localized within the candidate locus for tLBS5 on chromosome 5 (Fig.2) could be involved in Fe transport or subcellular Fe partitioning. Vacuolar ATP synthases are membrane localized proteins, which pump protons ($H^+$) across membranes to generate a charge gradient for the transport of ions across membranes (Finbow and Harrison 1997). The transport into storage compartments, such as the vacuole, represents an important mechanism for the sequestration of excess ions in plants including Fe (Becana et al. 1998; Schroeder et al. 2013).

The candidate genes for shoot Fe concentration included a casein kinase I (LOC_Os02g56560), which was located in the LD block surrounding the significant SNP on chromosome 2 (Fig.3). Casein kinases are evolutionary conserved eukaryotic protein kinases with multiple regulatory roles in plant metabolism and development (Lee 2009; Vidal et al. 2010). In rice, a casein kinase I (different from the one we identified) was shown to be involved in lateral root formation (Liu et al. 2003). Our own previous analyses suggested that the density of lateral fine roots was important in determining the capacity of releasing oxygen into the rhizosphere, which is required for Fe-oxidation and precipitation at the root surface, and thus represents an Fe exclusion mechanism (Wu et al. 2014).

Regarding candidate genes located in the peak region on chromosome 1 (Fig. 3), a potassium ($K^+$) channel (LOC_Os01g45990) putatively involved in potassium transport could be involved in Fe tolerance. Previous studies had shown that $K^+$ availability mitigated toxic effects of Fe on rice plants by limiting the translocation from roots to shoots (Li et al. 2001).

Among all identified candidate loci, we studied in more detail the two adjacent regions associated with tLBS5 located at around 28.5 Mb on chromosome 1. This region co-localized with several previously reported QTL reported from bi-parental QTL mapping experiments (Dufey et al. 2014; Wu et al. 2014), despite the otherwise low degree of co-localization of QTLs obtained from different populations and experimental systems. Although the sensitive haplotype at this locus (TGC) is relatively rare in the association panel (Fig. 2E), it is represented in widely grown mega-varieties such as IR64, which provides scope for adaptive breeding through replacement with the tolerant alleles. The significant SNPs of this region were

localized in two LD blocks, both of which contained genes putatively involved in detoxification and redox homeostasis. One was an arabino-lactone oxidase domain containing protein (LOC_Os01g49360) located in the first LD block. This class of enzymes has been shown to be involved in AsA biosynthesis (Smirnoff et al. 2001). However, since we did not observe significant differences in total AsA concentrations between contrasting haplotypes for significant SNPs on chromosome 1 (Table 1), we rejected the hypothesis of this gene mediating Fe tolerance via AsA biosynthesis. The second LD block of this region contained two GSTs (LOC_Os01g49710 and LOC_Os01g49720), which had previously been listed by Dufey et al.( 2014) as two out of 31 candidate genes in this chromosomal region, where multiple QTLs co-localized. GSTs constitute a large family of proteins with multiple functions, including detoxification of xenobiotic compounds, stress responses, responses to auxins, and signaling (Edwards and Dixon 2005; Laborde 2010; Marrs 1996). Both genes were up-regulated due to Fe toxicity in the eight genotypes investigated (Fig.4), although they were not listed among the significantly regulated genes in a previously published microarray study (Quinet et al. 2012), in which a single rice genotype (I Kong Pao) had been exposed to Fe stress. However, the transcriptional regulation was not associated with tolerance, since no differences in mRNA expression levels were observed between tolerant and sensitive haplotypes (Fig.4). In contrast, sequence analyses revealed polymorphisms between tolerant and intolerant haplotypes, which, in the case of LOC_Os01g49710 caused substantial modifications in the predicted protein structure. Because GSTs were suggested to be involved in oxidative stress response (Edwards and Dixon 2005), we tested the activity of the ascorbate-glutathione cycle in contrasting haplotypes, which constitutes a major component of plants' ROS detoxification machinery (Noctor and Foyer 1998). Haplotype differences were most pronounced in DHAR activity (Table 1), which constitutes one of the potential functions of plant GSTs (Edwards and Dixon 2005). That lower DHAR activity was associated with tolerance seems counter-intuitive, since high antioxidant activity is typically expected to confer tolerance to environmental stresses. In the case of this enzyme restoring the reduced form of ascorbate from dehydroascorbate (Noctor and Foyer 1998), it must be considered that reduced ascorbate may not only scavenge ROS, but also reduce $Fe^{3+}$ to $Fe^{2+}$, - a reaction, which is used in widely adopted ascorbate analyses assays (Gillespie and Ainsworth 2007; Ueda et al. 2013). Consequently, the presence of $Fe^{2+}$ may stimulate the Fenton reaction leading to the production of the hydroxyl radical, which cannot be scavenged effectively in plant cells (Apel and Hirt 2004). Thus, in the case of Fe toxicity, the presence of reduced AsA may have a detrimental rather than a protective effect. Based on the above converging evidence, the two GSTs are emerging as plausible candidate genes, which need to be further characterized to address the following questions: (i)

whether their involvement in tolerance to Fe-toxicity can be confirmed using reverse genetic approaches, and (ii) whether they directly have DHAR activity, or modulate DHAR activity indirectly, *e.g*. via signaling.

## 4.6 Conclusions

Screening of 329 accessions representing the entire genetic diversity of rice demonstrated that both exclusion and inclusion mechanisms are relevant under an acute short-term Fe stress. GWAS detected multiple loci associated with Fe toxicity tolerance, some of which co-localized with QTL identified previously using bi-parental mapping populations. For these results to make an impact on adaptive rice breeding, the following points need to be considered. (i) The screening protocol aimed at simulating an acute Fe stress during the vegetative growth stage. In the field, Fe toxicity may occur during different growth stages in a barely predictable manner, depending on variable environmental factors such as rainfall. Given the inconsistencies often observed between screening experiments for Fe toxicity tolerance in different environments, further analyses should focus on loci that have repeatedly been reported under different environmental conditions, such as the locus at 28-29 Mb on chromosome 1. Further testing of selected genotypes in different Fe toxic environments may also increase the level of confidence for the selection of suitable donors and traits for breeding. (ii) The loci identified in this study will only be useful for improvement of widely grown cultivars if these carry sensitive alleles at the proposed loci. Further analyses of allelic variants in widely grown mega-varieties are therefore warranted. (iii) The candidate genes nominated in this study need to be verified and functionally characterized in isogenic background. Reverse genetic studies are therefore warranted. Together these efforts will contribute to the breeding of more adapted cultivars and a better understanding of Fe toxicity tolerance mechanisms in rice.

## Acknowledgements

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# Chapter V. General Discussion

## 5.1 The impact of Fe toxicity on rice plants

The adverse effects of excess Fe in plant cells are mainly caused by the Fenton reaction leading to the generation of the extremely toxic hydroxyl radicals (OH·) that cannot be effectively scavenged by known antioxidants *in planta* (Apel and Hirt 2004). Through the DAB staining, $H_2O_2$ was visualized in the leaf blades not only under Fe toxic conditions but also under control conditions (Fig. 1B, **Chapter III**). The production of $H_2O_2$ predominately occurs during photosynthesis and photorespiration and to a lesser extent during the respiration processes (Slesak et al. 2007), which indicates that the generation of $H_2O_2$ is inevitable in plants. With the presence of free catalytic $Fe^{2+}$, the hydroxyl radicals will be generated and attack lipids, proteins and nuclear acids leading to irreversible damage (Becker and Asch 2005). The necrosis spots (cell death) on rice leaves can directly limit the photosynthesis rate. Moreover, moderate Fe stress affected photosynthesis through stomatal limitation while severe Fe stress inhibited the light-utilization through both stomatal and non-stomatal limitations (Pereira et al. 2013). Additionally, Fe toxicity has other adverse effects on plant growth. We selected one sensitive genotype IR29 and three tolerant genotypes Pokkali, FL483 and FL510 from the IR29/Pokkali population (**Chapter II**). These genotypes were grown in two treatments including the control as a luvisol (Meckenheimer Krume, Germany) and Fe toxicity as the additional supply of 1,000 ppm $Fe^{2+}$ in the soil. Both Pokkali and FL483 showed significantly higher tolerance than IR29 (Fig. 1A), which was consistent with the results in hydroponics screening. FL510 showed relatively lower leaf bronzing score than IR29, but the difference was not significant. When comparing the shoot K, Zn and Mn content between different treatments, significant differences were observed in all genotypes (Fig. 1B,C,D). Thus, we conclude that Fe toxicity not only affects rice growth by damaging the leaf cells and limiting the photosynthesis but also adversely affects the uptake of other nutrients such as K, Zn and Mn. Similar results such as phosphorus (P) uptake limitation under Fe toxicity were also reported by De Dorlodot et al. (2005) and Tanaka et al. (1966). The limiting effects might be caused by the damaged root structures and the Fe precipitates on root surface (**Chapter I**, Fig. 3B). In this context, Fe toxicity frequently co-occurred with other stresses leading to the complexity of adverse conditions or problem soils. To breed more adapted rice cultivars for Fe toxic fields, other limiting factors should also be taken in consideration. Interestingly, Pokkali showed significantly higher shoot Mn concentration than other genotypes (2.5-fold in control and 2.1-fold in Fe treatment) in our soil experiment (Fig. 1D). Pokkali thus can be considered as one promising genotype to study the interactions

between rice and Mn, for example to identify the tolerance mechanisms to Mn deficiency or toxicity in rice.

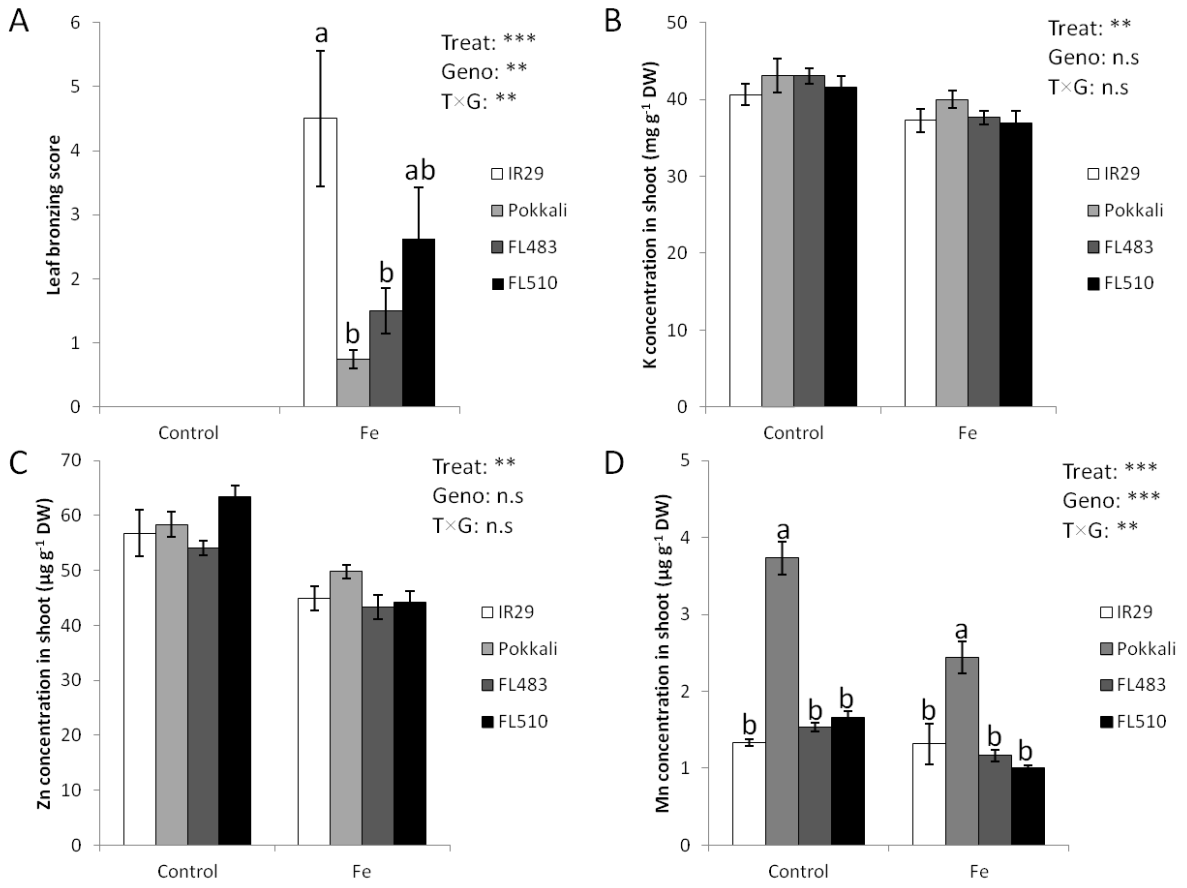


Figure 1. Screening of IR29, Pokkali, FL483 and FL510 genotypes in two different Fe treatments: normal luvisol from Meckenheimer Krume, Germany and luvisol with 1,000 ppm $Fe^{2+}$ supply for 2 weeks. (A) Leaf bronzing score, (B) shoot potassium, (C) zinc and (D) manganese concentration was measured. Data was analyzed with ANOVA investigating treatment, genotype and treatment by genotype interaction effect. Bars indicate mean values with standard error (n=4). The different letters above the data bars represent the difference of average values at a significant level of $P$ <0.05 with post-hoc multiple comparison test (LSD). Treat, treatment; Geno, genotype; T×G, treatment by genotype interaction; **, $P$ <0.01; ***, $P$ <0.001; n.s, not significant.

## 5.2 The screening conditions and phenotypes for Fe toxicity tolerance

Fe toxicity in rice can occur in various conditions regarding soil Fe content (20-5,000 ppm) and the timing of toxicity occurrence (from 2 weeks after transplanting until late reproductive stage) (Becker and Asch 2005). In the early vegetative growth stage, stressed plants exhibit reddish spots on leaves and decreased tiller number. When Fe toxicity occurred in the late vegetative or reproductive stages, it would delay the heading and flowering and produce thin and narrow panicles with decreased spikelet fertility (Ponnamperuma et al. 1955). Thus, in the different stages of Fe toxicity, suitable phenotypes should be chosen for the screening the tolerance. In this thesis, I mainly focused on the 5-day acute Fe toxicity (1,000 ppm) stimulating the type of Fe stress occurred in inland valleys receiving run-off water with high content of Fe from adjacent slopes. It occurred in the rice early vegetative stages in West Africa (e.g. Guinea, Ivory Coast) or Southeast Africa (e.g. Madagascar) (Becker and Asch 2005). The screening system is shown in Fig. 2. Nutrient solutions with/without 1,000 ppm $Fe^{2+}$ were applied in the screening to avoid other factors leading to contradictory tolerance rankings that were frequently observed in the same genotypes under different field conditions (**Chapter II**). The rhizosphere of each single plant was isolated (Fig. 2B) to prevent the scenario when roots of certain genotypes with high root-oxidizing power (e.g. Pokkali and FL510) tangled with others thus affecting the oxidation of $Fe^{2+}$ on root surface leading to biased performance. More importantly, $N_2$ gas was percolated in the nutrient solution for 15 minutes every 2 hours to maintain low redox potential, which limits the re-oxidation of $Fe^{2+}$ and accelerates stress symptoms expression (Fig. 2A) (Wang et al. 2008).

In the screenings of a large number of genotypes (QTL study, GWAS), a time-efficient and reliable method is essential. Leaf bronzing score (LBS) was consistently applied through this thesis due to the following reasons. First of all, LBS can be quickly assigned to individual plants and consistent among different tests. Secondly, it was widely adopted in many previous studies, such as bi-parental QTL mapping for Fe tolerance (Wu et al. 1997; Wan et al. 2003a; Wan et al. 2003b; Dufey et al. 2009). Thirdly, LBS showed significant agronomy relevance, which was negatively correlated to rice yield loss (Audebert and Fofana 2009). Moreover, LBS was also assigned to rice plants subjected to other oxidative stresses, such as Zn deficiency and $O_3$ stress (Höller et al. 2014; Ueda et al. 2014). Thus, it might also be useful in evaluating the tolerance to complex stress conditions in the field, e.g. co-occurrence of Fe toxicity with Zn deficiency or Fe toxicity with $O_3$ stress. To standardize the symptoms scoring for Fe toxicity in the early vegetative growth of rice, the typical pictures for different LBS were shown in supplementary Figure S2 (**Chapter II**).

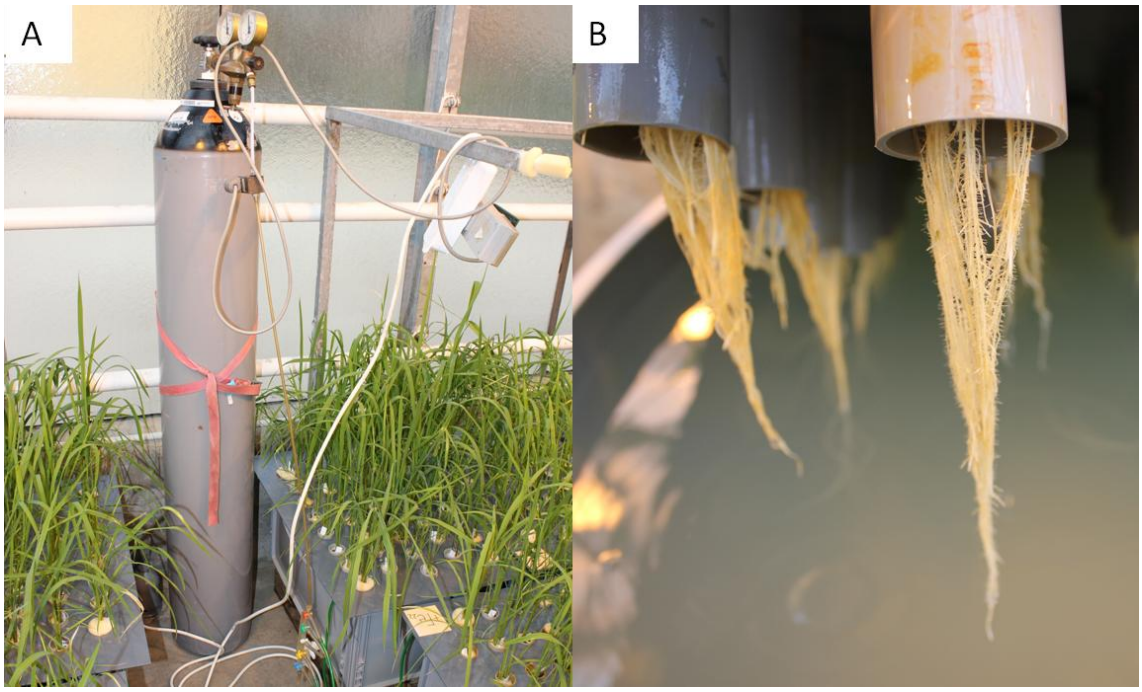


Figure 2. The screening system for Fe toxicity tolerance in this thesis. (A) $N_2$ gas was percolated into nutrient solutions for 15 minutes every 2 hours. (B) The roots of each single plant were separated using the PVC-tube fixed beneath the lids.

### 5.3 Genetic aspects of tolerance to Fe toxicity in rice

QTLs associated with the tolerance to Fe toxicity were identified by a number of studies summarized in **Chapter II**. Among the QTLs identified in multiple studies employing different mapping populations (Backcross Inbred Lines, BILs; Chromosome Segment Substitution Lines, CSSLs; $F_2$ population; Double Haploid; DH population; Recombinant inbred lines, RILs), genetic maps (RFLP, AFLP, SSR, SNP markers), stress intensities (250, 600 or 1,000 ppm $Fe^{2+}$) or rice growth stages, the co-localization analysis of the QTLs might provide an insight of the common tolerance loci that are promising for the breeding of tolerant cultivars. However, we should be cautious that the traits chosen must be responsive to Fe toxicity. These traits can be leaf bronzing symptoms (Wu et al. 1997; Wu et al. 1998; Wan et al. 2003a; Wan et al. 2003b; Shimizu et al. 2005; Wan et al. 2005; Dufey et al. 2009), relative shoot/root dry weight (Wu et al. 1997; Dufey et al. 2009), shoot Fe concentration (Wu et al. 1998; Shimizu et al. 2005; Shimizu 2009), antioxidants content or antioxidant enzymes activity (Wu et al. 1998). However, the independent shoot/root dry weight ratio, which was utilized by Wan et al. (2003a; 2003b) might not be an appropriate trait for Fe tolerance mapping or co-localization analysis. For the discussion of the genetic aspects of Fe tolerance, I only focused on the leaf bronzing symptoms and shoot Fe concentration.

In **Chapter IV,** 3 and 1 SNP markers were found to be significantly associated with leaf bronzing score (-Log *P*-values >4.0) on chromosome 1 and 5, respectively. The previously detected QTLs were localized based on the physical positions of the marker intervals and compared with GWAS results (Fig. 3). On chromosome 1, multiple loci were identified with leaf bronzing symptoms (Fig. 3A) indicating the complex genetic architecture of the tolerance. However, by comparing the physical positions of the marker intervals or associated SNP markers, some clusters were indeed detected. The most frequently identified chromosomal region is 26.739 to 29.385 Mb. The top three significantly associated SNP markers (id1046591, id1016614 and id1016768) completely overlapped with the interval of RM443 and RM403 detected by Wan et al. (2005). The other two regions showing co-localization were detected at the interval of 33.053 to 36.734 Mb (**Chapter II** and by Wan et al. (2005) and 37.713 to 41.541 Mb and by Wu et al. (1997). On chromosome 5, only one SNP marker, id500074 was significantly associated with leaf bronzing symptoms in **Chapter IV**, and no co-localization was identified with any previously reported QTL (Fig. 3B). For the trait of shoot Fe concentration, one significant SNP marker, id1015380 at the position of 26.118 Mb on chromosome 1 was detected close to one QTL between 26.739-27.335 Mb (Dufey et al. 2009) (Fig. 4A). On chromosome 2, the significant SNP, id2015632 was not co-localized with any reported QTL (Fig. 4B).

The frequently detected regions on chromosome 1, especially between 26.739 and 29.385 Mb, are certainly the most promising loci for the fine mapping of tolerance to various Fe toxic conditions. The co-localizations between the significant SNPs and other QTLs indicated that GWAS indeed is a useful tool for investigating the complex genetic architecture of Fe tolerance. GWAS detected some loci that were not found in QTL studies indicating the ability of GWAS in surveying a large range of natural variation in a single experiment. Moreover, GWAS showed higher resolution than QTL mapping, such as for leaf bronzing symptoms, the range of targeted loci was narrowed down to around 220 Kb suitable for gene discovery. Compared with GLM method, MLM incorporating population structure (PCA) (Price et al. 2006) and relative kinship largely reduces false positives due to the confounding from population structure (GLM results not shown). However, reducing the confounding effects of population structure might also reduce the detection of true positives correlated with sub-populations, which may explain why some QTLs were not co-localized with any significant SNPs in GWAS. Other explanations might be that the QTLs are linked with alleles that are rare in diversity panel (MAF <0.05) or where the phase of the allelic associations differ across sub-populations. This phenomenon might be common because of the significant evolutionary differences between *japonica* and *indica* sub-species, as described in **Chapter I**.

Combined bi-parental QTL mapping and GWAS has already been proven useful in identifying genetic basis of flowering time in *Arabidopsis* (Zhao et al. 2007; Brachi et al. 2010) and the tolerance to Al toxicity in rice (Famoso et al. 2011). It is also true for identifying tolerance loci to Fe toxicity in rice through mitigating the disadvantages from each single approach.

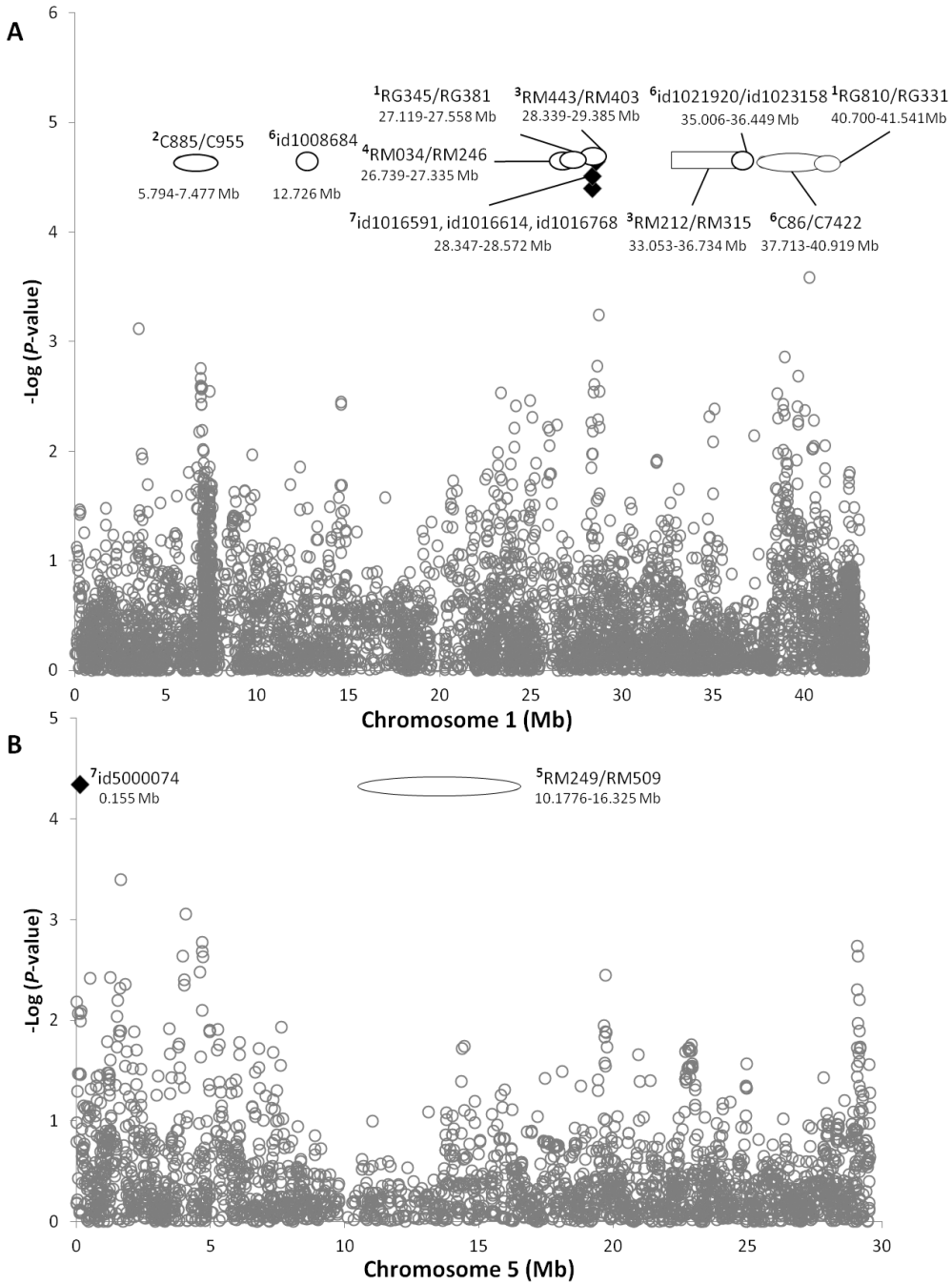


Figure 3. Co-localization analysis of the tolerance loci identified in GWAS and QTL mapping with the trait of leaf bronzing symptoms. The loci were shown with linked markers and physical positions (Mb) on (A) chromosome 1 and (B) chromosome 5. The significant association between SNP and phenotype was determined as -Log *P*-value >4.0. Small grey cycle represents single SNP marker. Different superscript numbers indicate different studies. [1] Wu et al. (1997); [2] Wan et al. (2003); [3] Wan et al. (2005); [4] Dufey et al. (2009); [5] Dufey (2015); [6] Chapter II; [7] Chapter IV.

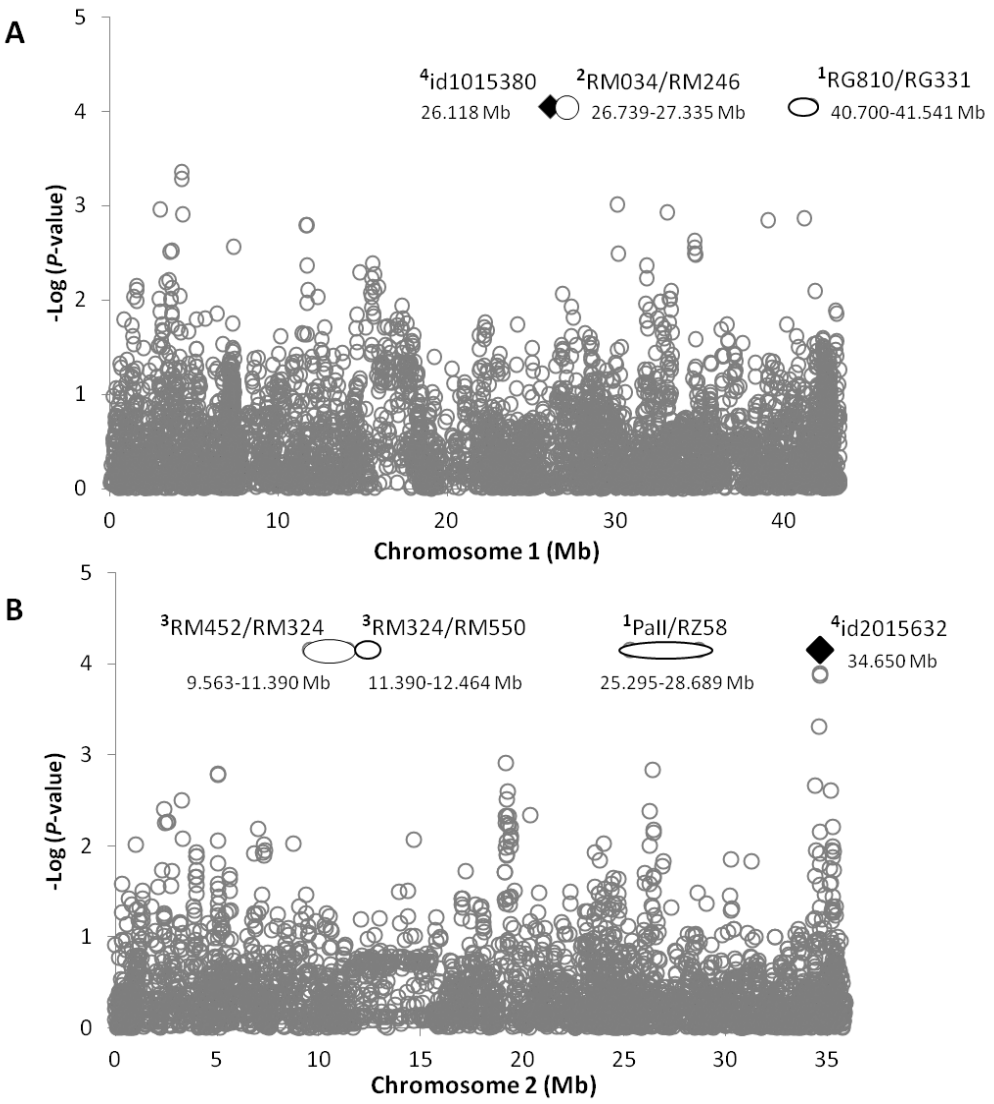


Figure 4. Co-localization analysis of the tolerance loci identified in GWAS and QTL mapping with the trait of shoot Fe concentration. The loci were shown with linked markers and physical positions (Mb) on (A) chromosome 1 and (B) chromosome 2. The significant association between SNP and phenotype was determined as -Log *P*-value >4.0. Small grey cycle represents single SNP marker. Different superscript numbers indicate different studies. [1] Wu et al. (1998); [2] Dufey et al. (2009); [3] Dufey (2012); [4] Chapter IV.

## 5.4 Root- / shoot-based tolerance mechanisms to Fe toxicity

Root-based tolerance serves as the first defense line against excess Fe in soil (Green and Etherington 1977). Root exclusion due to the formation of the physical barrier, or root plaque, is the most direct and effective approach (Becker and Asch 2005). The tolerance in FL510 was directly linked to the high root oxidizing power including the radial oxygen loss (ROL) (Armstrong 1967), which was significantly correlated with the Fe concentration in root plaque (Wu et al. 2012). ROL represents the oxygen diffusion from root aerenchyma to rhizosphere through lateral fine roots (Colmer 2002). As shown in **Chapter II**, two tolerant genotypes, Pokkali and FL510 possess larger pith cavity diameter in shoot and larger primary root diameter (**Chapter II**, Fig. 4), which increased the absolute volume of aerenchyma for oxygen transport, than the sensitive genotype IR29. Meanwhile, much denser lateral fine roots were also observed in tolerant genotypes leading to rapid oxygen diffusion. Root plaque formation also functions in sequestering toxic elements (e.g. Cd, Pb, Al and As) and reduces the uptake into rice shoots (Chen et al. 2006; Ultra et al. 2009; Wu et al. 2012; Cheng et al. 2014). Another aspect of root oxidizing power is related to enzymatic oxidization (Armstrong 1967). One candidate enzyme involved in the process is rice ferric reductase oxidase (*OsFRO*), in which a mutation leading to high tolerance was identified in the screening of 4,500 mutant lines subjected in a 300 ppm Fe stress (Ruengphayak et al. 2015). The exclusion mechanism was also proposed as root membrane selectively reduced the passage of $Fe^{2+}$ through the Casparian strip leading to the exclusion of Fe in the root apoplast (Green and Etherington 1977). However, when $Fe^{2+}$ ions present at high concentration (> 50 ppm) or toxicity occurred at seedling stage, this tolerance mechanism is not likely to play a significant role (Becker and Asch 2005), especially when the stress intensity is extremely high (e.g. 1,000 ppm). Other root-based tolerance mechanisms also include the immobilization and deposition of Fe in root (Becker and Asch 2005). In the roots of bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.), $Fe^{3+}$ pools were identified in the free space (Bienfait et al. 1985). However, the pools for the retention of excess Fe in roots have not been identified in rice yet. Moreover, under severe and sustained Fe toxic conditions, the retention in roots may not be sufficient for plants to maintain fitness.

Shoot-based tolerance mechanisms were investigated in both **Chapter III** and **IV** by transciptomic and biochemical analyzes. In **Chapter III**, we proposed three hypotheses regarding shoot-based tolerance in FL483: (i) Fe uptake, partitioning and storage, (ii) antioxidants scavenging the ROS produced by excess Fe and (iii) antioxidant enzymes and antioxidant-related enzymes for ROS scavenging. Ferritin, the ubiquitous Fe storage protein was found to play an important role to cope with Fe overload in mammal cells together with other two major proteins transferrin and heme

(Andrews 2000; Brissot et al. 2012). In plant cells, ferritin was considered as the main component for Fe storage together with the vacuole (Darbani et al. 2013; Moore et al. 2014). Stein et al. (2009) investigated the regulation of two rice ferritin genes in Nipponbare (ssp. *japonica*) and found that induced expression by excess Fe was only observed in rice leaves but not in roots. However, in **Chapter III**, the up-regulation of ferritin by Fe stress was only observed in roots (ssp. *indica*), which was in agreement with the findings a study where rice (I Kong Pao, ssp. *indica*) plants were subjected to a Fe stress (125 ppm) for 3 days (Quinet et al. 2012). Silveira et al. (2009) reported that Fe tolerance was positively correlated with the ferritin accumulation in shoot by comparing two varieties contrasting in Fe tolerance. However, the evidence was not strong enough to indicate that ferritin is a genuine tolerance factor to Fe toxicity because only one pair of genotypes was investigated. The different levels of ferritin accumulation between these two genotypes can be caused randomly, i.e., one genotype has a 50% chance of being higher than the other one. To test the possible role of ferritin in Fe tolerance, we obtained one activation tagged line (1E-04334) with a T-DNA insertion in the 5'-UTR region of ferritin (*OsFer1*) (Jeon et al. 2000). A mutant line, 1E-04334 and its wild-type Hwayoung (ssp. *japonica*) were exposed to a Fe stress of 1,000 ppm $Fe^{2+}$ for 5 days (Fig. 5A). The expression of *OsFer1* in shoot was highly induced by excess Fe in both Hwayoung and 1E-04334. The discordance of the regulation in ferritin gene in different tissues by excess Fe might be correlated to rice sub-species. 1E-04334 showed significantly higher expression level than the wild-type in Fe toxic conditions (Fig. 5B), but the stress symptoms between these two lines did not differ significantly after 5-day Fe treatment (Fig. 5C). It was concluded that the transcriptional regulation of ferritin did not account for the shoot tolerance to acute Fe toxicity. However, we cannot reject the possible role of ferritin in Fe tolerance because the Fe stress intensity in our experiments was extremely high. The typical shoot Fe concentration after a 5-day treatment can reach up to 9 mg $g^{-1}$ dry weight (**Chapter IV**). It might be far beyond the capability of ferritin to accommodate such high amounts of Fe in cells. The tolerance to other Fe stress types, for example chronic stress with lower Fe concentration, might be achieved by ferritin accumulation, which is warranted for further investigation.

The transcriptomic and enzymatic analyses showed that the tolerance in FL483 was associated with low ascorbate redox state, which was controlled by both DHAR and AO activities (**Chapter III**). Similar results were found in GWAS, where different tolerance in contrasting haplotypes was linked to different ascorbate redox state and DHAR activity. Höller et al. (2015) investigated the connection between total ascorbate and MDA concentrations in rice plants subjected to various stresses. The ascorbate level was negatively correlated with MDA concentration in control, Zn

deficiency or $O_3$ stress, but not for plants grown in Fe toxic conditions. Conclusive results suggested that the redox state rather than absolute amount of ascorbate played an important role in shoot-based tolerance; and ascorbate served as a pro-oxidant rather than an anti-oxidant. The findings were further confirmed by foliar ascorbate spray that aggravated the oxidative stress in Fe toxic conditions (**Chapter III**, Fig. 6D). In both **Chapter III** and **IV,** the DHAR activity differences were not highly correlated with the expression of DHAR genes (LOC_Os06g12630 and LOC_Os05g02530), but rather with the glutathione-S-transferase genes (LOC_Os01g49710, LOC_Os01g49720 and LOC_Os10g38780). Such discrepancy can be explained by the fact that DHAR genes indeed belong to the rice GST gene family, which possess multiple functions (Edwards and Dixon 2005; Liu et al. 2013). How the GST genes identified in this study regulate DHAR activity and what are the implications on Fe toxicity tolerance remain unknown. Thus, further investigations, for example using mutant lines (knock-out or over-expression) of these genes to look into the DHAR activity, are warranted.

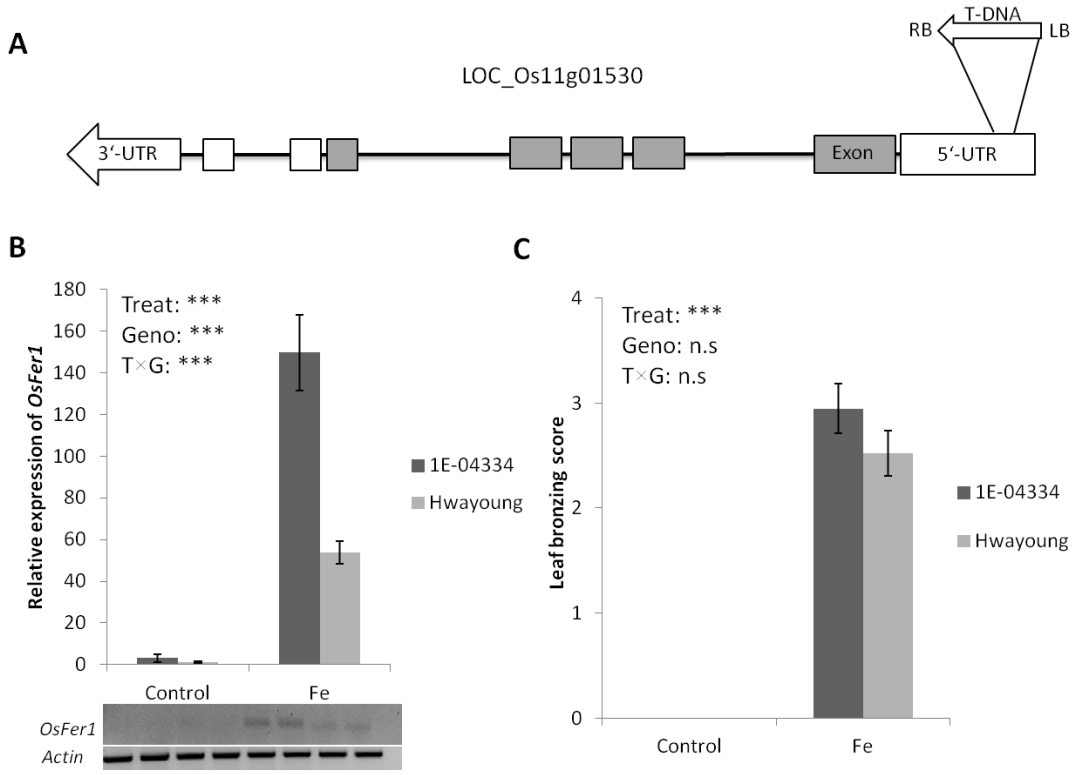


Figure 5. One T-DNA insertional activation tagged line, 1E-04334 with the wild-type Hwayoung was exposed to 1,000 ppm $Fe^{2+}$ for 5 days. (A) The schematic representation of the T-DNA insertion in the 5'-UTR region of *Os11g0106700* (*OsFer1*) in 1E-04334. (B) Relative expression of *OsFer1* in 1E-04334 and Hwayoung after 5-day Fe treatment. The expression of each sample was quantified using the ΔΔCT method. *Actin* was employed as endogenous reference gene and the average expression level of Hwayoung in control conditions was taken as the calibrator. Bars represent average values with standard errors (n=3). Two replicates of 1E-04334 and Hwayoung (from left to right) in control and Fe treatment were shown in the gel image. (C) Leaf bronzing score was employed as the stress symptom after 5-day Fe treatment. Bars indicate mean values with standard errors (n=12). Treat, treatment; Geno, genotype; T×G, treatment by genotype interaction; ***, *P* <0.001; n.s, not significant.

The major findings for root-/shoot-based tolerance in this thesis were summarized in Fig. 6. Root-based tolerance (exclusion) in certain genotypes (e.g. Pokkali and FL510) is conferred by root oxidizing power favored by shoot pith cavity and root aerenchyma together with large amount of lateral fine roots. Shoot-based tolerance is related to the low redox state to avoid the pro-oxidant property of reduced ascorbate that aggravates the oxidative stress. The low ascorbate redox state is achieved by DHAR alone or in combination with AO activity.

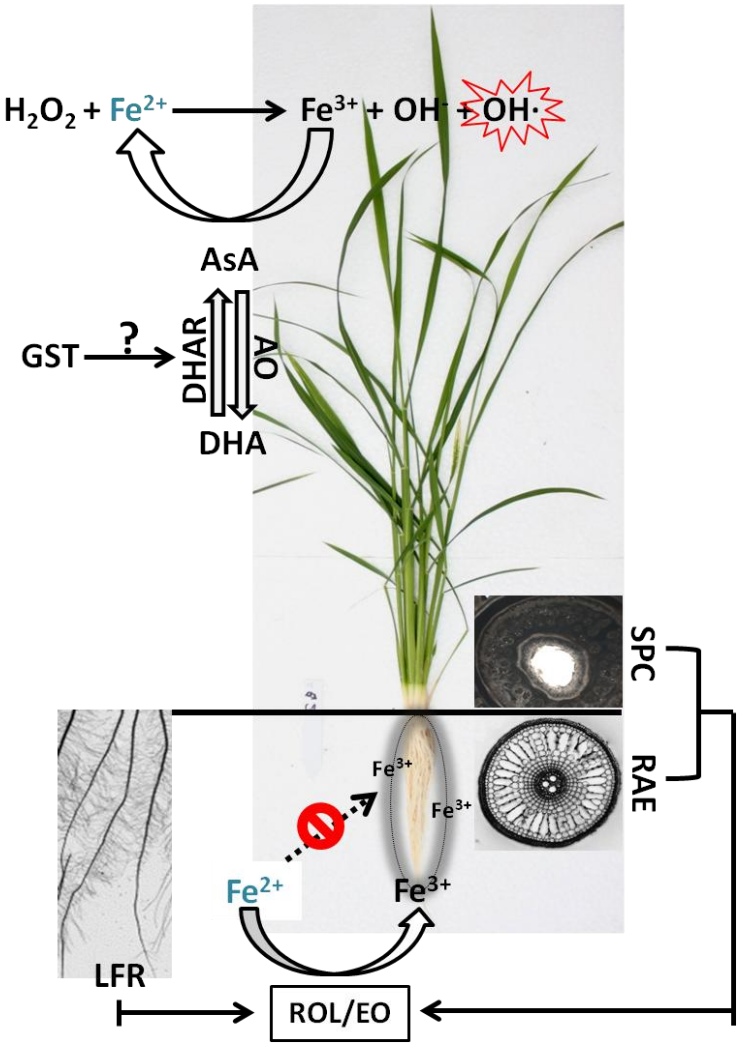


Figure 6. The conceptual model summarizing the root- and shoot-based tolerance identified in this thesis. The root-based exclusion mechanism is associated with the Fe plaque formation by oxidizing and precipitating $Fe^{2+}$ that is favored by shoot pith cavity, root aerenchyma and dense lateral fine roots. The shoot-based tolerance mechanism is related to the low ascorbate redox state, which avoids the reduction of $Fe^{3+}$ to $Fe^{2+}$ aggravating the oxidative stress. The low ascorbate redox state is controlled by DHAR and AO activities. GST genes possibly regulate the DHAR activity. *AO*, ascorbate oxidase; *AsA*, reduced ascorbic acid; *DHA*, dehydroascorbic acid; *DHAR*, dehydroascorbate reducatase; *EO*, enzymatic oxidation; *GST*, glutathione-S-transferase; *LFR*, lateral fine root; *RAE*, root aerenchyma; *ROL,* radial oxygen loss; *SPC*, shoot pith cavity.

## 5.5 Outlook

This study investigated the genetic and physiological factors of tolerance to Fe toxicity in rice utilizing multiple approaches including bi-parental QTL mapping, GWAS, transcriptomic and physiological analyses. Candidate tolerance loci were identified and distinct tolerance from root and shoot aspects were discovered. Still, many open questions remain to be exploited, which might be interesting and useful for better understanding of the tolerance to Fe toxicity and for breeding more adapted cultivars.

Several genes were proposed to be promising in enhancing Fe tolerance for future investigations. Two 4,5-DOPA-dioxygenase extradiol genes (LOC_Os01g65680 and LOC_Os01g65690) were identified in the co-localized region of 35-40 Mb on chromosome 1 (Fig. 3A). These two genes were annotated as the key enzymes for the synthesis of betalamic acid, which is the structural and chromophoric unit of antioxidant pigment, betalains (Gandía-Herrero and García-Carmona 2013). The enzymes also possess $Fe^{2+}$-binding capacity. Even though betalains do not exist in rice, the functions of 4,5-DOPA-extradiol-dioxygenase may contribute to the metabolism of other aromatic compounds possessing antioxidant capacity as revealed in studies on general plant resistance (Bahramnejad et al. 2010). One insertional knock-out mutant for LOC_Os01g65680, 1B-10813 was obtained and subjected to 1,000 ppm Fe stress together with co-segregating and independent wild-type lines (Fig. 7A). The knock-out mutant showed significantly less tolerance than wild-type lines (Fig. 7B). The sensitivity in 1B-10813 might be caused by the knock-out effect leading to (1) incapability of chelating excess $Fe^{2+}$ by the enzymes, or (2) inability to generate aromatic compounds that detoxify the ROS. Further investigations are warranted to illustrate the underlying mechanisms. Other two genes, an iron regulated transporter (*OsIRT1*), which is involved in the acquisition of Fe from the rhizosphere (Ishimaru et al. 2006), and a casein kinase I gene (LOC_Os02g56560) putatively involved in the lateral roots formation (Liu et al. 2003), are of interest for the investigations of root-based tolerance regarding Fe uptake and Fe exclusion. Potassium ($K^+$) availability in rice was found useful to mitigate the toxic effect of excess Fe by limiting the translocation of Fe from roots to shoots (Li et al. 2001). The $K^+$ channel gene (LOC_Os01g45990) detected near the significant SNP for shoot Fe concentration on chromosome 1 (Fig. 4A) might be involved in the tolerance by regulating $K^+$ transport.

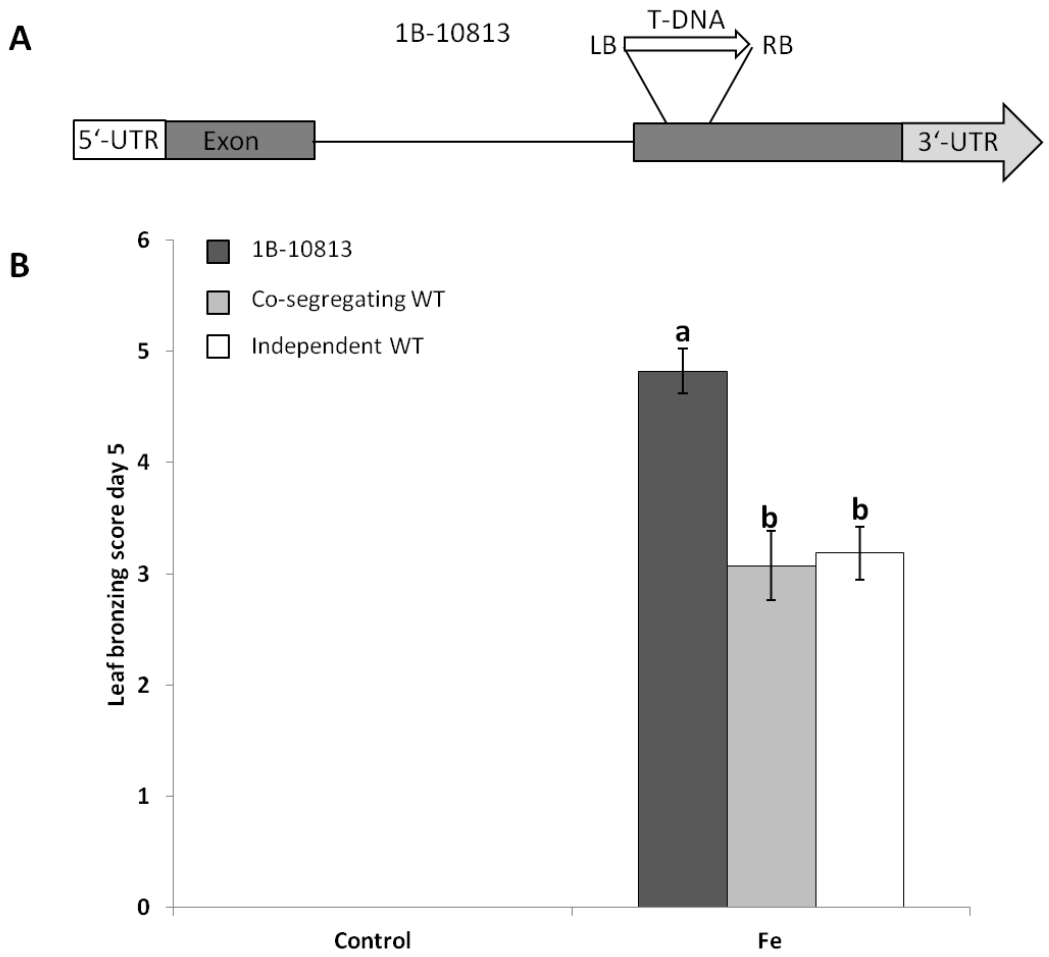


Figure 7. Screening of one T-DNA insertional knock-out line, 1B-10813 with the co-segregating wild-type (WT) and independent WT plants in 1,000 ppm Fe stress for 5 days (A) Schematic representation of the T-DNA insertion in the second exon of LOC_Os01g65680 in the mutant line. (B) Leaf bronzing score after 5-day treatment. Bars represent mean values ± standard error (n=3). Different letters above the data bars indicate significant differences at *P* <0.001.

The distribution of Fe was investigated at tissue and cellular scale (**Chapter III, Fig. 3, 4**). However, Fe distribution at the sub-cellular level was not achieved in this study due to the low resolution of the conventional histochemical staining methods. The vacuole is considered as an important component for excess toxic elements storage (Becana et al. 1998; Moore et al. 2014; Schroeder et al. 2013) and a putative vacuolar ATP-synthase (LOC_Os05g01560) was identified in the candidate locus for leaf bronzing symptom on chromosome 5 (Fig. 3B). Vacuolar ATP synthases are membrane localized proteins, which pump protons ($H^+$) across membranes to generate a charge gradient for the transport of ions such as $Fe^{2+}$ (Finbow and Harrison 1997; Kobayashi and Nishizawa 2012). The excess Fe stored in the vacuole may differ between genotypes contrasting in tolerance. Imaging the sub-cellular

localization of Fe, such as in vacuole, and measuring the *in situ* speciation are essential. Synchrotron-based techniques, e.g. X-ray fluorescence, X-ray absorption spectrometry, and mass spectrometry-based techniques, such as secondary ion mass spectrometry and laser-ablation inductively coupled mass spectrometry were previously utilized in revealing the distribution patterns of elements (Zhao et al. 2014). One high-sensitivity fluorescence probe, 7-(4-methylpiperazin-1-yl)-4-nitrobenz-2-oxa-1,3-diazole (MPNBD) was synthesized and employed in the imaging of Fe in *Arabidopsis* plants (Park et al. 2014). These techniques and chemicals might also be feasible for investigating Fe distribution at sub-cellular level.

To breed more adapted cultivars in Fe toxic fields, the common loci identified in both QTL mapping and GWAS, such as 26.7-29.4 Mb on chromosome 1, should be taken into consideration. The favorable traits for Fe tolerance are related to root oxidizing power, for example early formation of shoot and root aerenchyma and dense lateral roots. These traits are favorable in the formation of Fe plaque on root surfaces thus excluding further excess Fe uptake, and are also related to maintaining relatively low cellular ascorbate redox state through DHAR and/or AO activity regulation. However, because of the complex soil conditions in Fe toxic fields, there is still a long way to go to achieve this goal.

The Fe content in rice grains is relatively lower than in Maize, wheat or soybean, and IDA affects about 2 billion people worldwide (http://www.who.int/nutrition/topics/ida) especially in many Asian and African areas where rice is the main energy source (Masuda et al. 2013). The breeding of Fe bio-fortified cultivars is an effective, efficient and sustainable strategy to address the IDA problem (Aung et al. 2013). As described in **Chapter I**, a number of studies employing either single or multiple transgenic approaches reported the increased Fe content in rice grains. Concerning the research topics in this thesis, we would like to propose one question: can we take the advantages from the disadvantageous conditions in Fe toxic soils? In anaerobic, flooded rice fields, soluble Fe is abundantly available and could be tapped for bio-fortification of rice grains. A relatively high amount of Fe is taken up by rice plants but less or no toxicities occur, meanwhile, the excess Fe can be transported into rice seeds, that will be beneficial to increase yield production in Fe toxic areas and to the IDA affected people who rely on rice as staple food as well. However, to the best of our knowledge, no study has so far attempted to simultaneously address the problem of Fe toxicity and Fe bio-fortification. To achieve this goal, the main question is how to avoid the toxic effects from excess Fe. In this context, shoot-based tolerance, such as Fe partitioning and storage in old leaves (Tanaka et al. 1966) or in sub-cellular components (vacuole, ferritin and other Fe-binding proteins) is vital to address such a problem. Another concern is how to effectively load high amount of Fe into rice grains. It can possibly be achieved by the regulation of Fe chelators and transporters

to increase the Fe flux from xylem and phloem to grains. Fe translocation inside plants is accomplished by various types of influx and efflux transporters (Kobayashi and Nishizawa 2012). After uptake from soil, Fe is chelated with citrate and transported in xylem by citrate efflux transporter (*OsFRDL1*), which might be useful to increase the grain Fe content (Sperotto et al. 2012). Due to the lack of xylem discontinuity at the base of each grain (Zee 1972), it is feasible to directly load Fe from xylem in vascular bundle to the nuclear and aleuron cells as previously proposed for Zn bio-fortification (Stomph et al. 2009). Nicotianamine (NA) plays an important role in Fe chelating and transport in phloem, with the aid of *Yellow Stripe-1 Like* (YSL) transporter family (Koike et al. 2004; Aoyama et al. 2009; Ishimaru et al. 2010). One mutant line over-expressing *OsYSL15* showed increased Fe concentration in rice grains (Lee et al. 2009a) and the knock-down of *OsYSL2* decreased the seeds Fe concentration (Koike et al. 2004). Together with the findings of the increased Fe content in grains by over-expressing NA synthase genes (Inoue et al. 2003; Johnson et al. 2011; Lee et al. 2009b); both NA and YSL transporters play vital roles in transferring Fe from the phloem into aleuron cells. In rice grains, Fe is mainly distributed in aleurone layer and embryo. However, more than 70% of the microelements including Fe was removed from grains after polishing to white rice that is generally consumed (Sellappan et al. 2009). Fe fortification should be focused on the major sink of Fe in white rice - the endosperm. A number of studies reported that by over-expressing ferritin genes under the control of endosperm-specific promoters, such as *GluB-1* (glutelin) and *Gbl-1* (globulin), a 2- to 3-fold increase of grain Fe content was achieved (Goto et al. 1999; Vasconcelos et al. 2003).

Another common problem in micro-element bio-fortification is that the increased uptake of the target element often co-occurred with the uptake of other undesirable or even toxic elements. In the case of Fe bio-fortification, frequently observed undesirable ions are for example Cd, Co and Ni (Korshunova et al. 1999; Connolly et al. 2002). This problem can also be avoided /alleviated with two approaches: screening for Fe transporters with low toxic ion transport capabilities or altering of substrate specificity of transporters through genetic manipulation (Sperotto et al. 2012). Water management in rice field was also found to be effective in restricting Cd accumulation in grains (Kobayashi and Nishizawa 2012).

The breeding of adapted cultivars with desirable agronomic traits and beneficial grain Fe concentration will be an important objective for Fe toxic environments. The goal might be achieved through exploiting the diverse natural variations in rice and a better understanding of molecular basis of the Fe homeostasis.

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# Supplementary Data

## Chapter II.

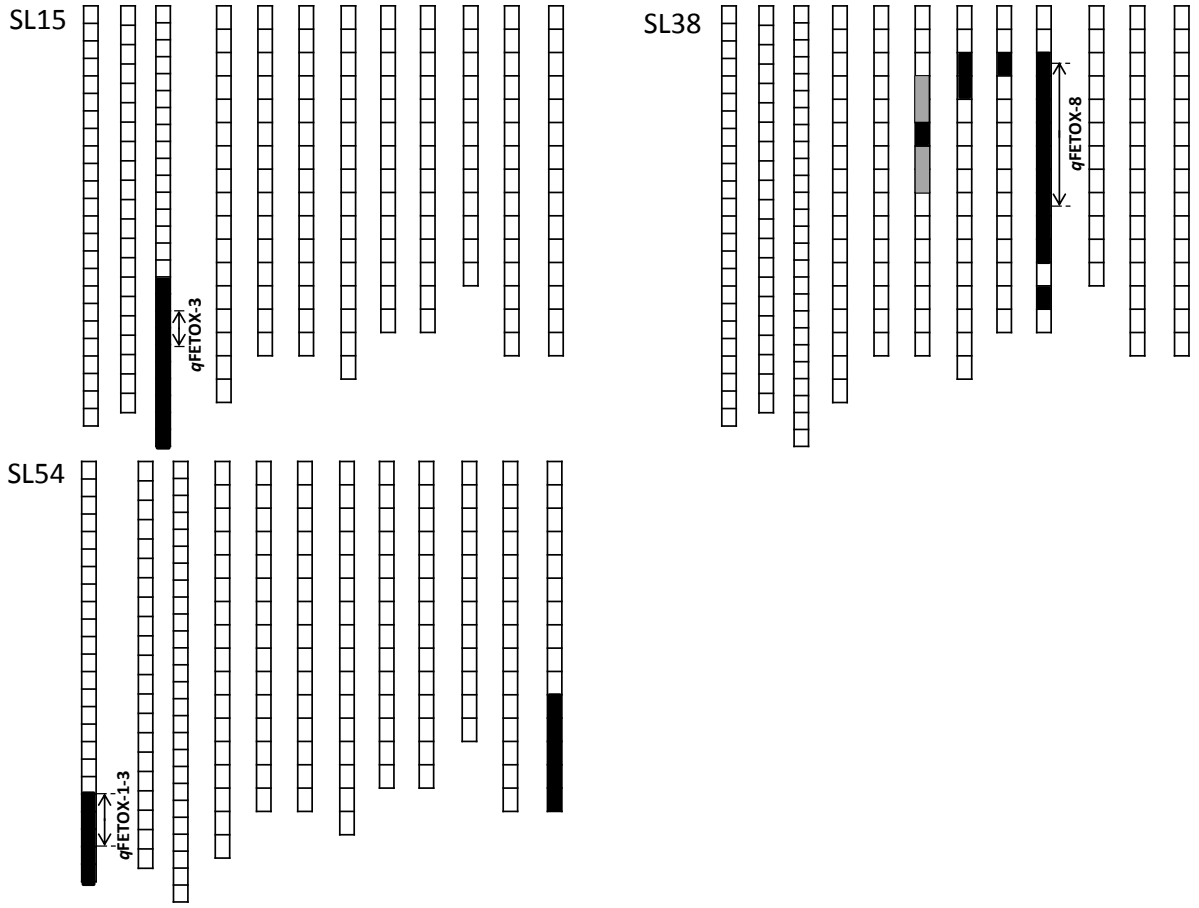


**Figure S1.** Schematic representation of the genotypes of chromosome segment substitution lines (SL), SL15, SL38 and SL54. Black and white bars represent Kasalath and Nipponbare segments, respectively. Grey bars indicate heterozygous segment. Approximate positions of three putative QTLs detected in Nipponbare / Kasalath population were shown. The effect of *qFETOX-1-3* was to decrease leaf bronzing score and *qFETOX-3* and *qFETOX-8* can increase the leaf bronzing score.

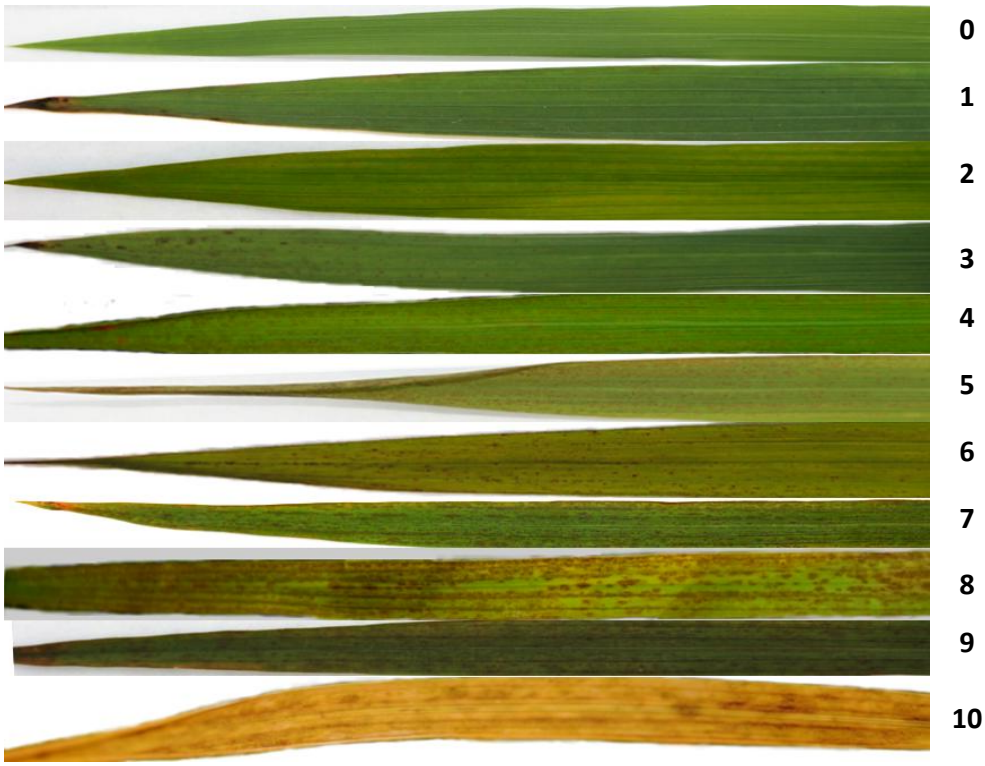


**Figure S2.** Representative photos of leaf bronzing scores ranging from 0 (healthy leaf) to 10 (dead leaf).

## Chapter III.

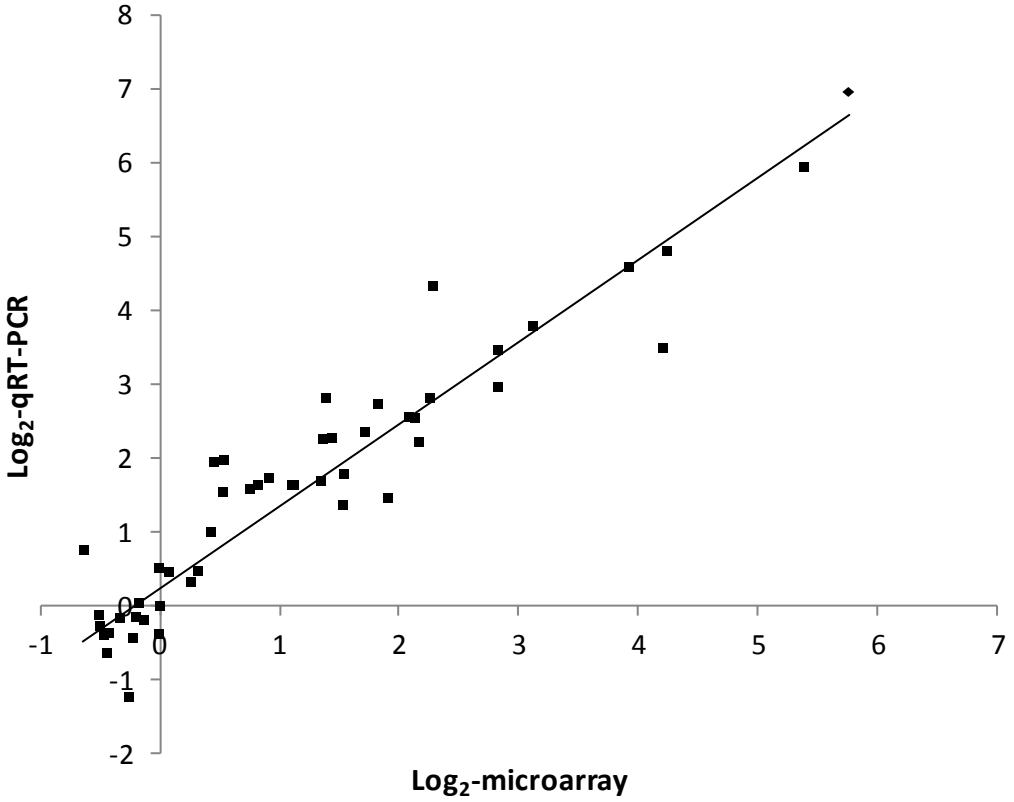


**Figure S1.** Validation of microarray data by qRT-PCR with selected genes in roots and shoots. Data from both microarray and qRT-PCR were normalized by setting the expression level of IR29 in the control as 1, and log to the base 2 of these relative expression levels were plotted. The primers for selected genes and complete expression patterns in microarray and qRT-PCR are given in Supporting Information Data S2.

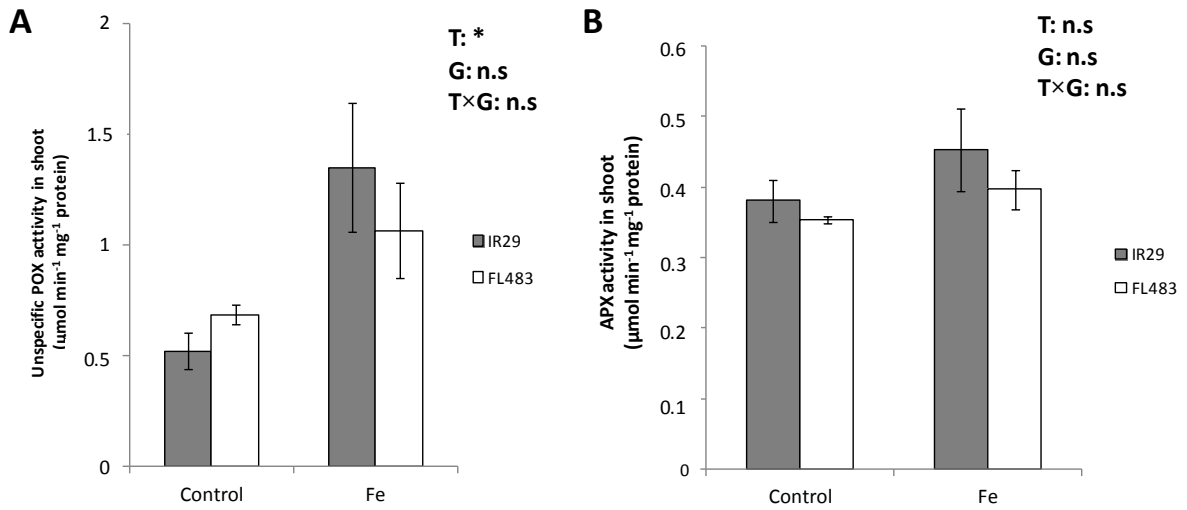


**Figure S2**. Additional biochemical characteristics in different tissues of contrasting rice genotypes IR29 and FL483 exposed to Fe toxicity (1,000 ppm $Fe^{2+}$ for 4 days) (A) Unspecific peroxidase (POX) activtity, (B) ascorbate peroxidase (APX) activity in shoot. Bars represent mean values ± SE (n=3). T, treatment; G, genotype; *, *P* <0.05; ***, *P* <0.001; n.s, not significant.

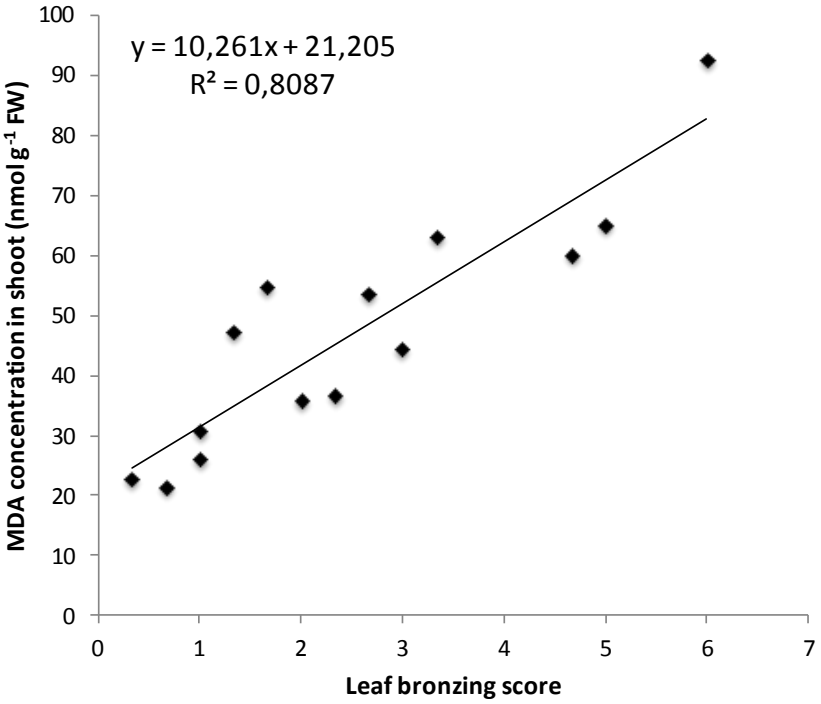


**Figure S3.** Correlation between Fe-stress induced leaf bronzing score and shoot MDA concentration analyzed in 14 individual plants of four different rice genotypes (sensitive IR29 and Kiang-Cho-Chiu and tolerant FL483 and Taichung-Native 1) after 4-day Fe treatment (1,000 ppm). The correlation was statistically significant at *P* <0.01 level (2-tailed).

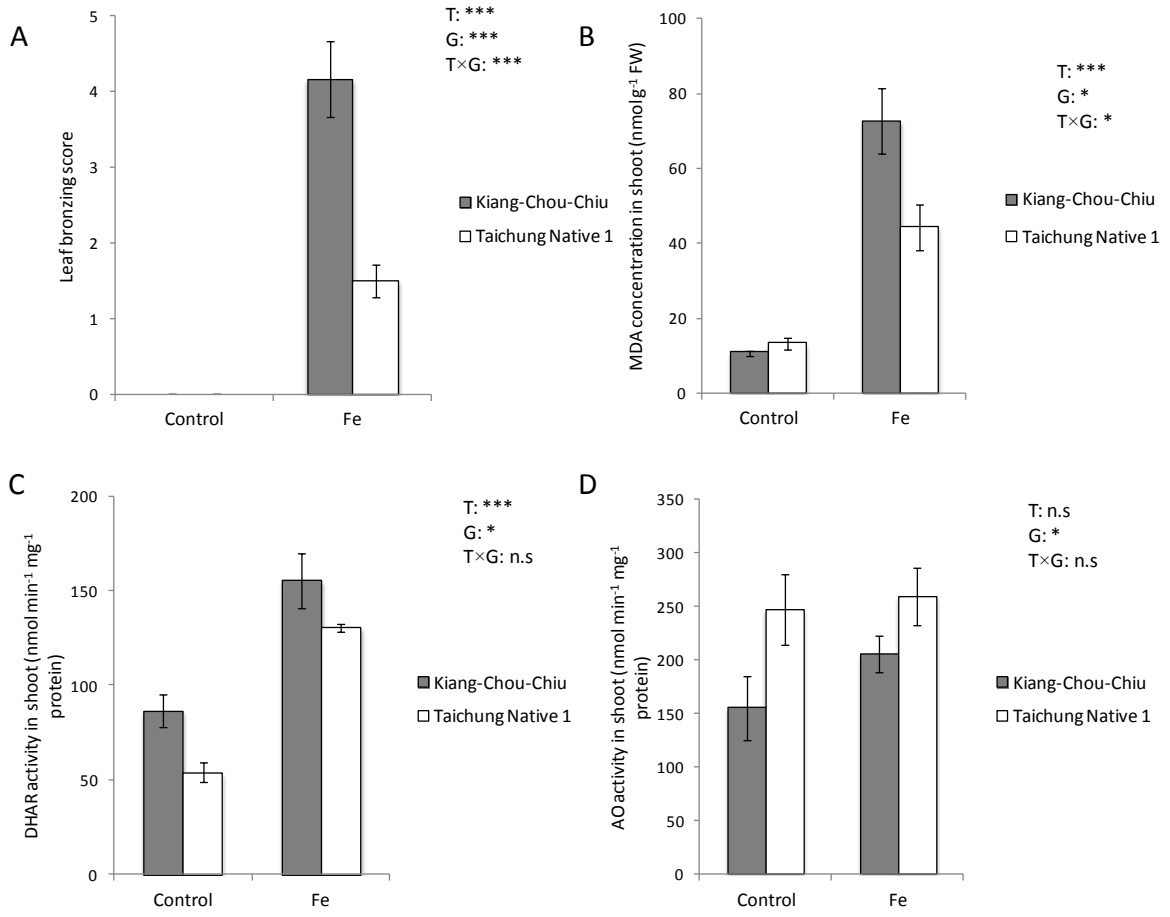


**Figure S4.** The responses of the sensitive genotype Kiang-Chou-Chiu and the tolerant genotype Taichun-Native 1 to 4-day Fe treatment (1,000 ppm). (A) Leaf bronzing symptoms, (B) shoot MDA concentration, (C) shoot DHAR activity and (D) shoot AO activity. Bars represent mean values ± SE (n=4). T, treatment; G, genotype; *, *P* <0.05; ***, *P* <0.001; n.s, not significant.

## Supplementary Data S1-S6

See 'Plant Cell and Environment. DOI: 10.1111/pce.12733 'Supporting Information, *pce12733-sup-0001-data_S1toS6.xlsx.'*

## Chapter IV.

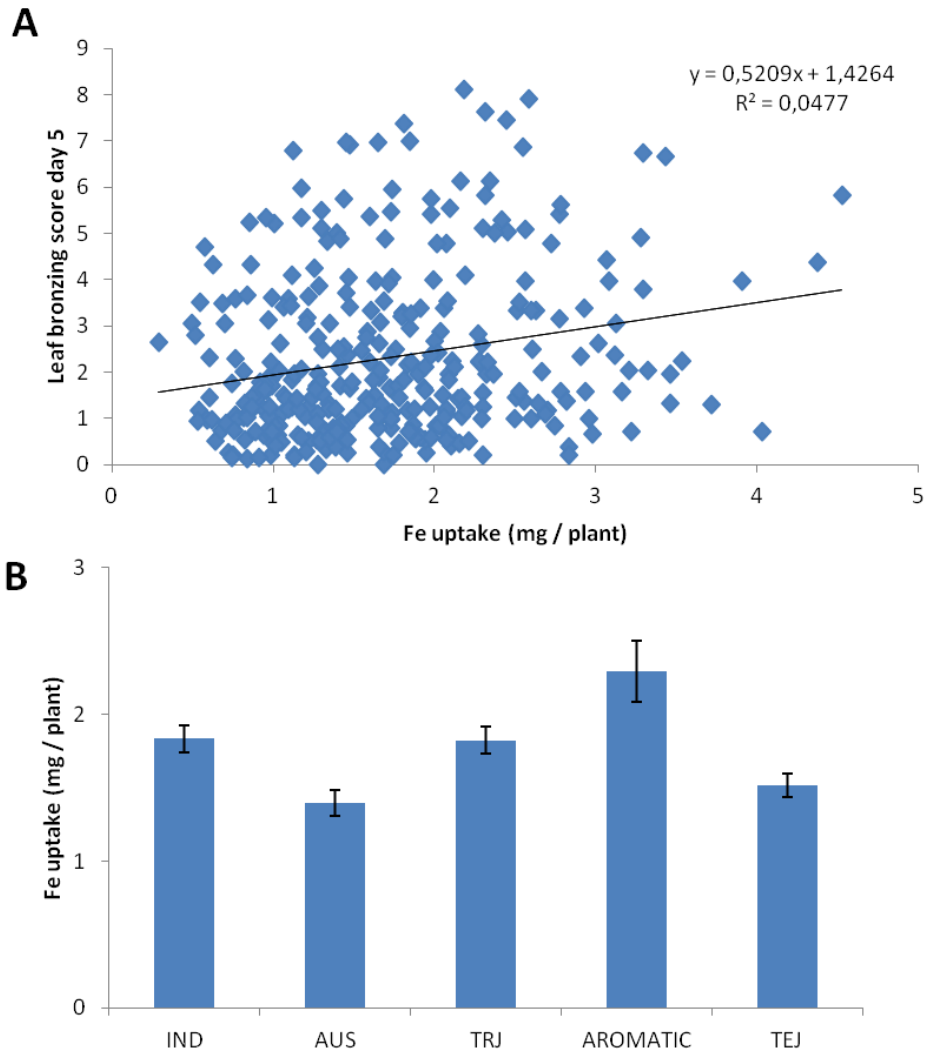


Figure S1: (A) Linear regression of shoot Fe uptake versus leaf bronzing scores (B) Sub-population analysis of shoot Fe uptake; IND indica, TRJ tropical japonica, TEJ temperate japonica. Data bars show mean values and standard errors; bars not sharing the same letter are significantly different at *P* <0.05 by Tukey's HSD test.

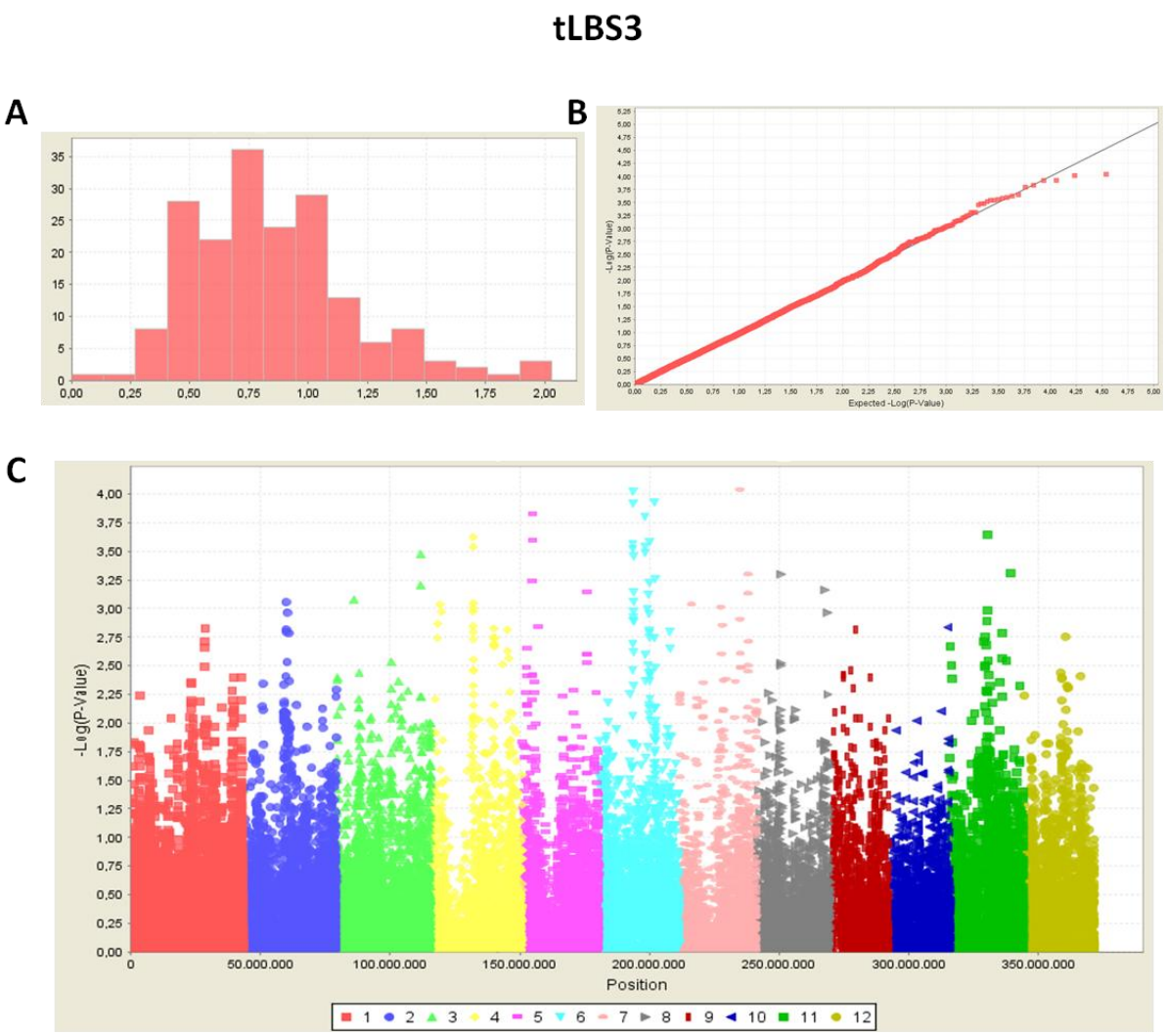


Figure S2: Association mapping results for root-transformed leaf bronzing score (tLBS3) after three days of iron treatment. (A) Frequency distribution of tLBS3 in the association panel. (B) q-q-Plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying *P*-values for each marker according to mixed linear model analysis. Colors encode different chromosomes.

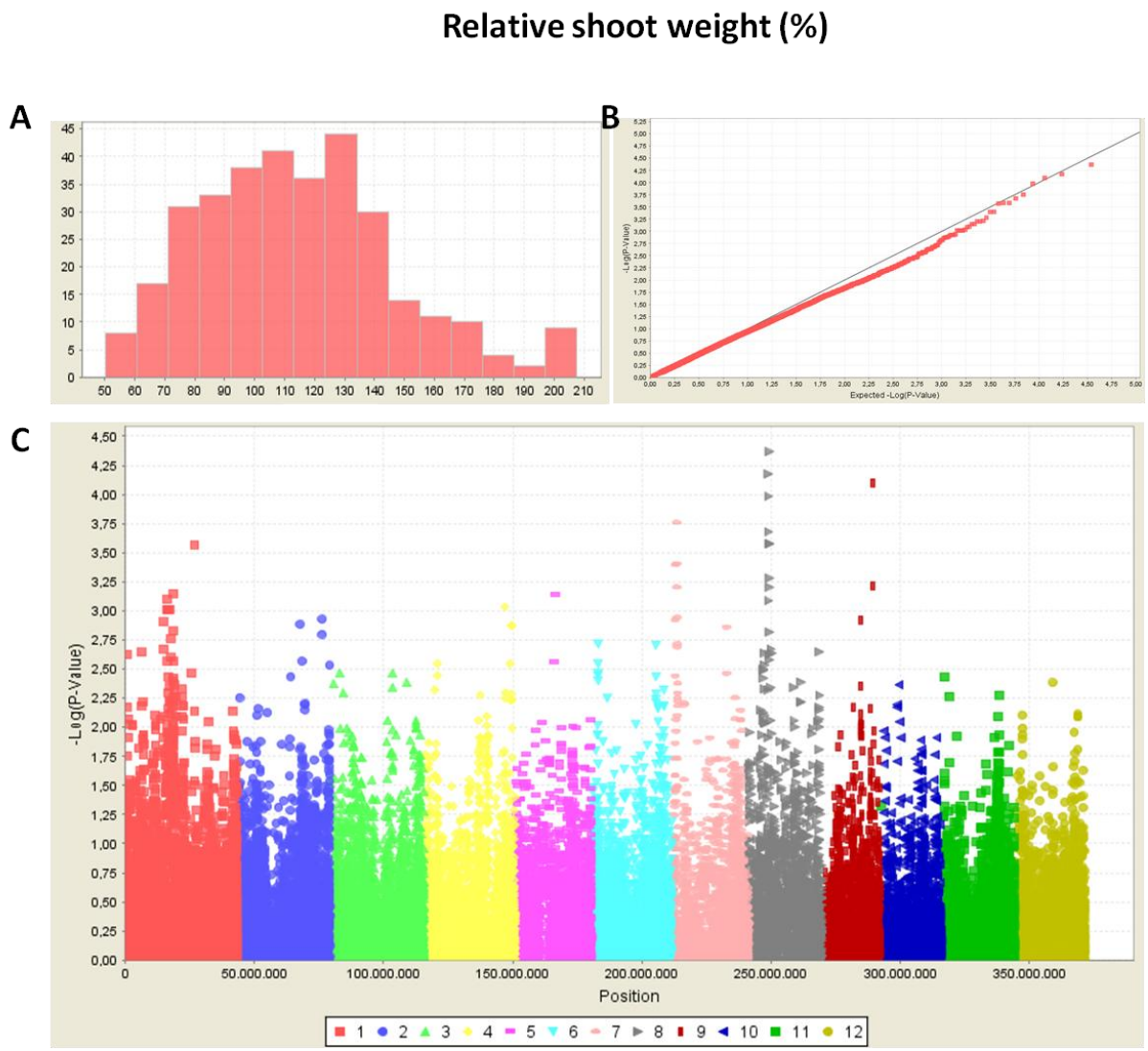


Figure S3: Association mapping results for relative shoot weight (as % of control) after five days of iron treatment. (A) Frequency distribution of relative shoot weight (%) in the association panel. (B) q-q-Plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying *P*-values for each marker according to mixed linear model analysis. Colors encode different chromosomes.

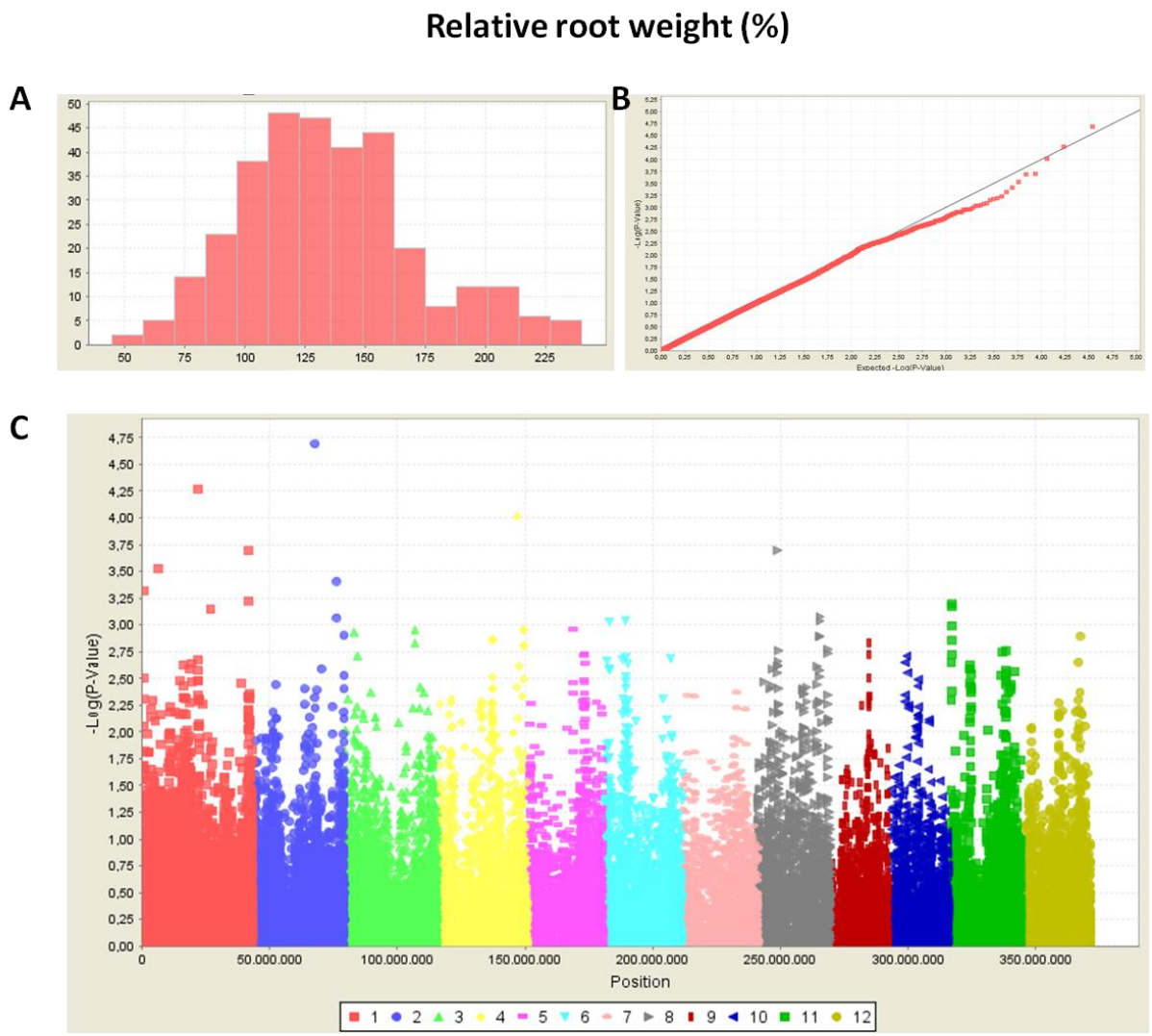


Figure S4: Association mapping results for relative root weight (as % of control) after five days of iron treatment. (A) Frequency distribution of relative root weight (%) in the association panel. (B) q-q-Plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying *P*-values for each marker according to mixed linear model analysis. Colors encode different chromosomes.

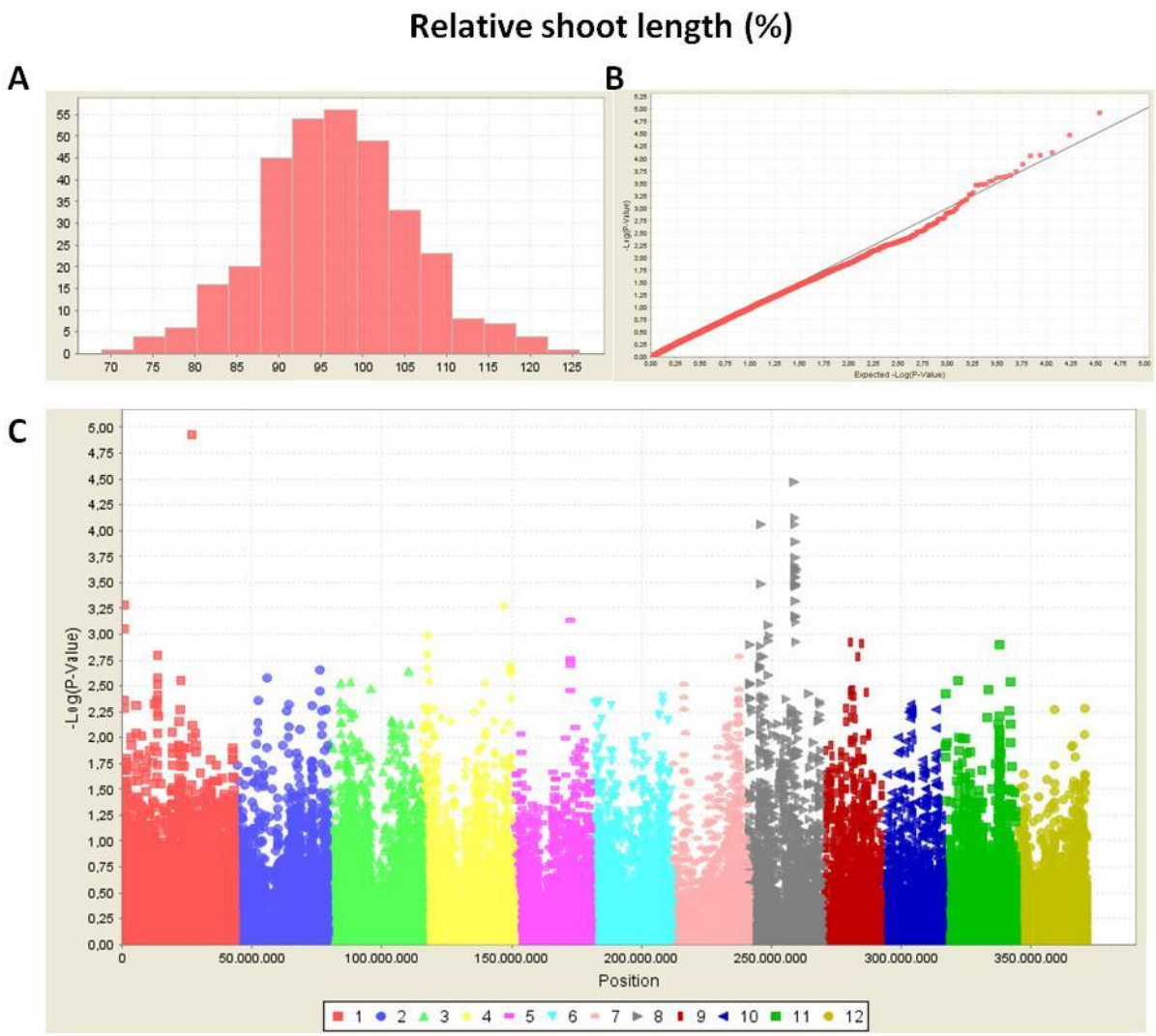


Figure S5: Association mapping results for relative shoot length (as % of control) after five days of iron treatment. (A) Frequency distribution of relative shoot length (%) in the association panel. (B) q-q-Plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying *P*-values for each marker according to mixed linear model analysis. Colors encode different chromosomes.

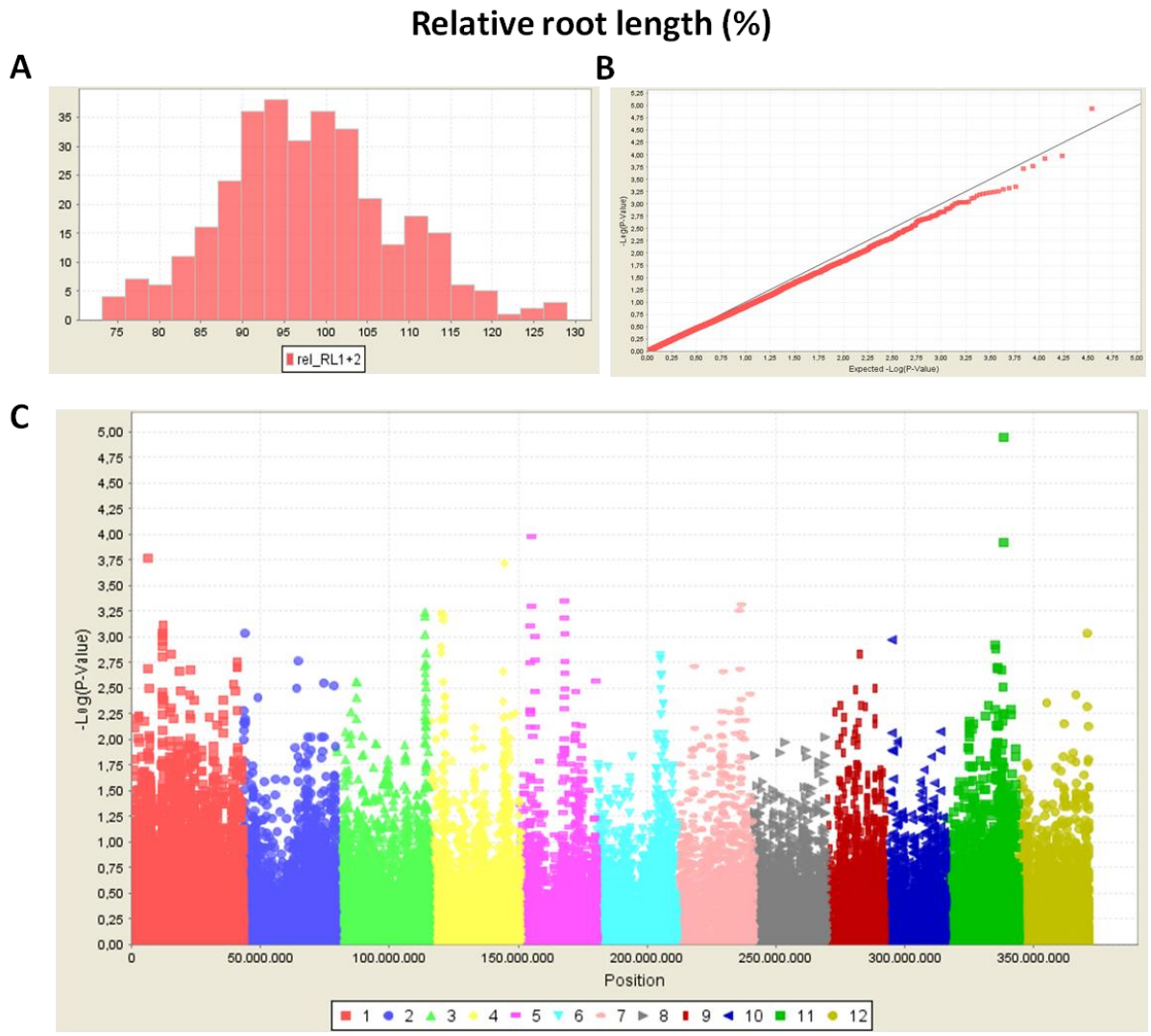


Figure S6: Association mapping results for relative root length (as % of control) after five days of iron treatment. (A) Frequency distribution of relative root length (%) in the association panel. (B) q-q-Plots comparing expected and observed *P*-values for marker-trait associations. (C) Manhattan plot displaying *P*-values for each marker according to mixed linear model analysis. Colors encode different chromosomes.

## Supplementary Data S7

See 'Theoretical and Applied Genetics, DOI:10.1007/s00122-015-2569-y, *Supplementary Data S7.*'