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A quick and efficient screen for resistance to iron toxicity in lowland rice

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Summary—Zusammenfassung

Iron (Fe) toxicity is a major stress to rice in many lowland environments worldwide. Due to excessive uptake of Fe^{2+} by the roots and its acropetal translocation into the leaves, toxic oxygen radicals may form and damage cell structural components, thus impairing physiological processes. The typical visual symptom is the "bronzing" of the rice leaves, leading to substantial yield losses, particularly when toxicity occurs during early vegetative growth stages. The problem is best addressed through genotype improvement, *i.e.*, tolerant cultivars. However, the time of occurrence and the severity of symptoms and yield responses vary widely among soil types, years, seasons, and genotypes. Cultivars resistant in one system may fail when transferred to another. Better targeting of varietal improvement requires selection tools improving our understanding of the resistance mechanisms and strategies of rice in the presence of excess iron. A phytotron study was conducted to develop a screen for seedling resistance to Fe toxicity based on individual plants subjected to varying levels of Fe (0–3000 mg L^{-1} Fe supplied as $Fe(II)SO_4$), stress duration (1–5 d of exposure), vapor-pressure deficit (VPD; 1.1 and 1.8 kPa), and seedling age (14 and 28 d). Genotypes were evaluated based on leaf-bronzing score and tissue Fe concentrations. A clear segregation of the genotypic tolerance spectrum was obtained when scoring 28 d old seedlings after 3 d of exposure to 2000 mg L^{-1} Fe in a high-VPD environment. In most cases, leaf-bronzing scores were highly correlated with tissue Fe concentration (visual differentiation in includer and excluder types). The combination of these two parameters also identified genotypes tolerating high levels of Fe in the tissue while showing only few leaf symptoms (tolerant includers). The screen allows selecting genotypes with low leaf-bronzing score as resistant to Fe toxicity, and additional analyses of the tissue Fe concentration of those can identify the general adaptation strategy to be utilized in breeding programs.

Key words: air humidity / leaf-bronzing score / *Oryza sativa* / selection tool / tolerance mechanisms / VPD

Eine schnelle und effiziente Methode zur Evaluierung von Eisentoxizitätsresistenz bei Nassreis

Eisen(Fe)-Toxizität ist weltweit einer der wichtigsten abiotischen Stressfaktoren in Nassreisproduktionssystemen. Bei übermäßiger Aufnahme von Fe^{2+} durch die Wurzel und seine akropetale Verlagerung in die Blätter kommt es zur Bildung toxischer Sauerstoffradikale, die vor allem Zellmembranen schädigen und so physiologische Prozesse stören. Das typische sichtbare Symptom dieser Schädigungen ist das so genannte „leaf bronzing", welches substanzielle Ertragseinbußen zur Folge hat, besonders, wenn Eisentoxizität in der frühen vegetativen Wachstumsphase auftritt. Die beste Strategie mit diesem Problem umzugehen, ist die Nutzung verbesserter, toleranter Genotypen. Allerdings interagieren Zeitpunkt des Auftretens und Intensität des Stresses sowie Auswirkungen auf den Ertrag stark mit Bodentyp, Jahr und Saison sowie Genotyp. Sorten, die in der einen Umwelt tolerant erscheinen, können in einer anderen versagen. Eine zielgerichtete Sortenanpassung benötigt Auswahlkriterien, die unser Verständnis der Resistenzmechanismen verbessern. Zur Entwicklung einer Methode zur Selektion widerstandsfähiger Reissetzlinge wurde eine Studie im Phytotron durchgeführt, bei der Einzelpflanzen unterschiedlichen Alters (14 und 28 Tage nach Aussaat) unterschiedlichen Fe-Angeboten (0–3000 mg L^{-1} Fe als $Fe(II)SO_4$), Stressperioden (1–5 Tage) und atmosphärischen Dampfdruckdefiziten (VPD: 1,1 and 1,8 kPa) ausgesetzt wurden. Die Genotypen wurden nach „leaf-bronzing score" und Fe-Gehalt im Gewebe bewertet. Eine klare Aufspaltung in das Toleranzspektrum der Genotypen wurde mit 28 Tage alten Setzlingen erreicht, die für 3 Tage bei hohem VPD 3000 mg L^{-1} Fe ausgesetzt waren. In den meisten Fällen war der Fe-Gehalt des Gewebes mit dem „leaf-bronzing score" signifikant positiv korreliert, wodurch eine visuelle Differenzierung zwischen „includer"- und „excluder"-Sorten erreicht wurde. Die Kombination dieser beiden Parameter ermöglichte es zudem, auch solche Genotypen zu erkennen, die bei hohen Fe-Gehalten im Gewebe kaum Blattsymptome zeigten (tolerante „includer"). Die hier vorgestellte Selektionsmethode ermöglicht es, Genotypen mit niedrigem „leaf-bronzing score" zu selektieren und im Anschluss durch Bestimmung der Fe-Gehalte im Gewebe deren Adaptationsstrategie zu erkennen und zu nutzen.

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1 Introduction

Fe toxicity is one of the most important abiotic stresses limiting rice production in lowland systems (*Dobermann* and *Fairhurst*, 2000). It has been reported to widely occur in several Asian countries, including China, India, Indonesia, Thailand, Malaysia, and the Philippines. In West Africa, it is widespread throughout the humid forests and Savanna zones, affecting 30%–60% of the cultivated lowland area (*WARDA*, 2001). Rice yields are reportedly reduced by 12%–100%, depending on the severity of toxicity and the tolerance of the rice cultivars (*Benckiser* et al., 1982; *Sahrawat* and *Diatta*, 1996; *Audebert* and *Sahrawat*, 2000). In flooded soils, oxygen is rapidly depleted by the respiration of microorganisms and plant roots. Therefore, shortly after the inundation of a rice field, reduction of Fe oxides and hydroxides can result in the accumulation of large amounts of Fe^{2+} in the soil solution, given an Fe-toxic precondition (*Ratering* and *Schnell*, 2000). This process is particularly pronounced in acid sulfate soils (*Prade* et al., 1993) or in inland swamps and irrigated lowlands with light textured soils, high extractable acidity, and low fertility (*Benckiser* et al., 1982).

Iron toxicity is a nutrient disorder which is brought about by the uptake of Fe^{2+} to concentrations exceeding 300 mg kg^{-1} (*Tanaka* and *Yoshida*, 1972; *Yamauchi and Peng,* 1995) that disrupt or overexpress a number of metabolic processes, resulting in damage of the rice plant (*e.g.*, *Bienfait*, 1985; *Bode* et al., 1995). Commonly observed symptoms are rusty leaf spots (bronzing), stained leaf edges, and a dark brown and poorly developed root system (*Dobermann* and *Fairhurst*, 2000).

There is no clearly established relationship between the severity of Fe toxicity, symptoms expressed, and yield. These relationships may vary among crop developmental stages within the cropping season as well as among seasons and years. Average reported yield losses due to Fe toxicity are in the range of 12%–35% (*Lantin* and *Neue*, 1989). However, Fe-induced yield losses and leaf bronzing were more pronounced in a dry- as compared to a wet-season crop (*Sahrawat* and *Diatta*, 1996), whereas, Fe-induced yield reductions of up to 30% have been reported to occur without any foliar symptoms (*Abifarin*, 1988). Generally, crop damage is largest when toxicity occurs at the seedling and early vegetative growth stages of rice, in the worst case leading to a complete failure of the crop (*Abu* et al., 1989; *Abraham* and *Pandey*, 1989). A number of resistance strategies to Fe toxicity in rice have been proposed: (1) exclusion of Fe^{2+} at the root level, thus avoiding Fe^{2+} damage to the shoot tissue *via* rhizospheric oxidation and root ion selectivity (*Chen* et al., 1980a, b), (2) inclusion and avoidance *via* internal compartmentation of Fe^{2+}, *e.g.*, apoplastic immobilization or preferential storage in old leaves or photosynthetically less active leaf sheath tissue (*Tadano*, 1975; *Smith*, 1984; *Audebert* and *Sahrawat*, 2000), and (3) inclusion and tolerance *via* increased thresholds to elevated levels of Fe^{2+} within leaf cells, probably through enzymatic detoxification in the symplast (*Bienfait*, 1985; *Gupta* et al., 1993; *Thongbai* and *Goodmann*, 2000).

Even though some knowledge to the mechanisms underlying those strategies exists, to date screening tools for Fe-toxicity tolerance in rice are exclusively based on leaf symptoms and yield (*e.g.*, *Gunawardena* et al., 1982; *Akbar* et al., 1987; *Mahadevappa* et al., 1991) but not on actual resistance mechanisms such as exclusion or tolerance. This may partly be due to the fact that the physiological mechanisms proposed are difficult to investigate and thus are not suited for mass-screening methods. The objective of this paper is to develop a standardized method to screen a large number of rice genotypes for their response to Fe toxicity in the early vegetative stage, differentiating between and selecting for avoidance and tolerance traits.

2 Materials and methods

2.1 Experimental site, set-up, and growing conditions

Experiments were conducted at the Institute of Plant Nutrition of the University of Bonn in a climate chamber with a 12 h photoperiod, 300 mmol m^{-1} s^{-1} illumination (PAR), 50%/85% day/night relative humidity and 26°C/20°C day/night temperature, and an average day/night vapor-pressure deficit of 1.75/0.35 kPa. Rice plants were cultivated in Yoshida medium (hydroponic culture). It contained in full strength 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 $FeCl_3 \cdot 6H_2O$ [in mono-

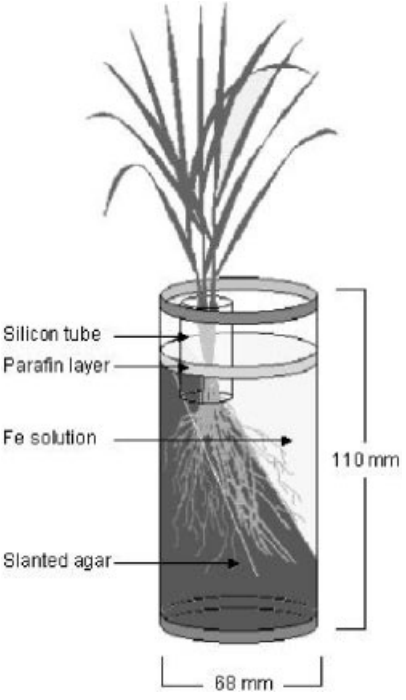

Figure 1: Schematic presentation of the set-up for Fe(II)-toxicity screening.
Abbildung 1: Schematische Darstellung des Anzuchtsystems zur Evaluierung von Eisentoxozitätsresistenz bei Nassreis.

hydrate citric acid]) with a pH of 5. Transparent polystyrol pots (Greiner bio-one, Bonn, Germany, 400 mL, 68 mm diameter, 110 mm height) were filled with 150 mL of half-strength Yoshida growth medium autoclaved with 1% Agar at 121° and 1200 hPa (Tuttnauer Systec Autoclave, ELV 3850). The medium was slanted and let to cool at a 45° angle. The slant agar pots were wrapped in aluminum foil and stored in a cold room at 4°C until further use.

Rice seeds were soaked over night in water and incubated and pregerminated on filter paper in petri dishes for 60 h in the growth chamber. Before transferring the 3 d old rice seedlings into the slant agar pots, a PVC tube of 15 mm diameter and 65 mm length was inserted to a depth of 25 mm into the agar slant in such a way that rice seedlings growing within the tube were protected from direct contact with the liquid medium. After the transfer of the rice seedling, the pots were filled with 150 mL half-strength Yoshida solution, covered with aluminum foil to exclude light in the root zone and placed for 2–4 weeks in the climate chamber (Fig. 1).

2.2 Plant materials

Seeds of lowland-rice cultivars (*Oryza sativa* L. and *O. glaberrima* Steud.), varying in the reported degree of sensitivity towards reduced Fe, were obtained from various sources. All genotypes used in this study are listed in Tab. 1, including their origin and the seed source.

2.3 Leaf scoring

Iron-toxicity responses were scored by subjective visual assessment of Fe-toxicity symptoms on fully expanded leaves (bronzing symptoms) for the entire plant and expressed as percentage leaf area affected. As scoring system, the Standard Evaluation System for scoring for leaf blast (*Pyricularia oryzae*) lesions provided by the International Network for the Genetic Evaluation of Rice (INGER; *IRRI*, 1996) was adapted for Fe toxicity as follows (percentage leaf area affected = score): 0 = 0 (no symptoms); 1–9 = 1; 10–29 = 3; 30–49 = 5; 50–69 = 7; 70–89 = 9; 90–100 = 10 (dead leaf).

2.4 Iron content in plant tissues

At each sampling, leaves were scored for Fe-toxicity symptoms and the aboveground biomass was harvested. Samples were oven-dried at 70°C to constant weight, weighed, and analyzed for tissue Fe content by atomic-absorption spectrometry (Perkin-ELMER AAS 1100B, Überlingen, Germany), following a hot pressure digestion with saturated ammonium nitrate solution at 180°C for 7 h and a subsequent filtering and standard dilution to 100 mL.

2.5 Iron-stress and air-humidity treatments

To determine the concentration of Fe^{2+} required to visually differentiate between sensitive and tolerant rice cultivars, Fe concentrations were varied for Fe-toxicity symptoms to be

Table 1: Characteristics of the rice genotypes used in the study. Abbreviations: sat—*Oryza sativa*; gla—*Oryza glaberrima*; jap—*japonica*; ind—*indica*; Trad—traditional; Impr—improved. Country codes: CI—Côte d'Ivoire; GH—Ghana; LI—Liberia; NI—Nigeria; PH—Philippines; SE—Senegal; SL—Sierra Leone. IITA: International Institute for Tropical Agriculture. INGER: International Network for Germplasm Evaluation of Rice in Africa. IRRI: International Rice Research Institute. SARI: Savannah Agricultural Research Institute. WARDA: West Africa Rice Development Association (now: The Africa Rice Center).
Tabelle 1: Eigenschaften der in der Studie verwendenten Nassreisgenotypen. Abkürzungen: sat – *Oryza sativa*; gla – *Oryza glaberrima*; jap – *japonica*; ind – *indica*; Trad – traditionell; Impr – verbessert. Länder-Abkürzungen: CI – Côte d'Ivoire; GH – Ghana; LI – Liberia; NI – Nigeria; PH – Philippinen; SE – Senegal; SL – Sierra Leone. IITA: International Institute for Tropical Agriculture. INGER: International Network for Germplasm Evaluation of Rice in Africa. IRRI: International Rice Research Institute. SARI: Savannah Agricultural Research Institute. WARDA: West Africa Rice Development Association (jetzt: The Africa Rice Center).

Cultivar	Spp	Sub Spp	Country of origin	Trad/ Impr.	Seed source	Fe-sensitivity
CG14	gla		SE	Trad	INGER	moderate
CK4	sat	ind	SL	Impr	WARDA	tolerant
I Kong Pao	sat	ind	NI	Impr	WARDA	moderate
IR12979-24-1	sat	ind	PH	Impr.	IRRI	sensitive
IR31785-58-1-2-3-3	sat	ind	PH	Impr.	WARDA	sensitive
IR4630-22-2	sat	ind	PH	Impr	WARDA	sensitive
ITA 306	sat	ind	NI	Impr	IITA	moderate
ITA 320	sat	ind	NI	Impr	IITA	moderate
MR123	sat	jap	GH	Impr	IITA	sensitive
Sahel 108	sat	ind	SE	Impr	WARDA	tolerant
Sikamo	sat	ind	NI	Trad	IITA	sensitive
Suakoko 8	sat	ind	LI	Trad	INGER	tolerant
Tox4004-8-1-2-3	sat	ind	NI	Impr	SARI	tolerant
WITA7	sat	ind	CI	Impr	SARI	tolerant

induced in sensitive genotypes whereas no symptoms were to occur in tolerant genotypes. The genotypes IR31785-58-1-2-3-3 (sensitive) and Suakoko8 (tolerant) were used for this experiment (Tab. 1). At 14 and 28 d after sowing, Fe (applied as $FeSO_4$) was added to the culture solution to final concentrations of 0, 1000, 2000, 3000 mg Fe^{2+} L^{-1}, respectively. The nutrient/Fe solution was covered with 3 mL of liquid paraffin, creating a 3 mm diffusion barrier for oxygen to maintain a sufficiently low redox potential to prevent the oxidation of Fe^{2+} to Fe^{3+}. Seedling leaves were assessed visually for the expression and the severity of Fe^{2+}-toxicity symptoms (bronzing) at daily intervals for up to 5 d after Fe addition. Thereafter, the seedlings were harvested for dry biomass and the determination of tissue Fe content. Parallel to the Fe treatments, seedlings of Suakoko8 and Sahel108 (both tolerant) were subjected to two levels of vapor-pressure deficit (VPD) during the photoperiod (1.75 kPa and 1.11 kPa). Vapor-pressure deficit was reduced by evaporation from a water bath into a transparent plastic frame containing the pots. Humidity in the enclosure was monitored with a TinyTag Plus Moisture Meter (Gemini Data Loggers, Part No: TGP – 1500+ West Sussex, UK). Leaf symptoms of Fe toxicity were scored at 1, 2, and 3 d after treatment application. At 17 and 31 d after sowing, plants from all treatments were sampled.

2.6 Validation of the screening method

Fourteen rice genotypes of various origin and with known field behavior under Fe-toxicity conditions including the three used in the previous experiments (Tab. 1) were grown as described above. At 14 and 28 d after sowing (DAS), all plants were treated with two levels of Fe^{2+} (0 and 2000 mg L^{-1}) at ambient VPD of 1.75 kPa for 3 d. Thereafter, leaf symptoms were scored, plants were sampled, and tissue Fe content was determined.

3 Results

3.1 Effect of external Fe concentration on leaf symptoms and Fe(II) uptake

Root systems of 14 and 28 d old seedlings of tolerant Suakoko8 and sensitive IR31785-58-1-2-3-3 were exposed for 5 d to various external Fe^{2+} concentrations in the nutrient solution ranging from 0 to 3000 mg L^{-1}. Only about half of the roots were exposed to the Fe treatments as the other 50% were growing in the nutrient-rich agar. Suakoko8 had a significantly ($p < 0.05$) larger biomass than IR31785-58-1-2-3-3 in all cases, but addition of Fe to the medium had no influence on dry-matter accumulation in either genotype (data not shown). Roots of both genotypes showed brown coatings with the color intensity increasing with increasing Fe^{2+} concentration of the culture solution. With exposure to 3000 mg L^{-1} Fe^{2+}, root damage occurred after 4 d in the 4-week-old seedlings and after 3 d in the 2-week-old seedlings of both genotypes.

In both genotypes, Fe^{2+}-toxicity symptoms on leaves increased as a function of both concentration and duration of exposure, independent of seedling age (Fig. 1). However, leaf symptoms in tolerant Suakoko8 were always less pronounced than in sensitive IR31785-58-1-2-3-3; even at the highest external concentration. Symptom scores ranged from 1 to 7.8 and from 1 to 8 in the 2-week-old seedlings of Suakoko8 and IR31785-58-1-2-3-3; respectively. In the 4-week-old seedlings, scores ranged from 1 to 9 and from 1 to 9.5 in Suakoko8 and IR31785-58-1-2-3-3, respectively. The largest difference in leaf-bronzing score between the two genotypes was observed for both seedling ages at 3 d after application of 2000 mg L^{-1} Fe^{2+} (score of 6 in 2- and of 3.3 in 4-week-old seedlings, Fig. 2).

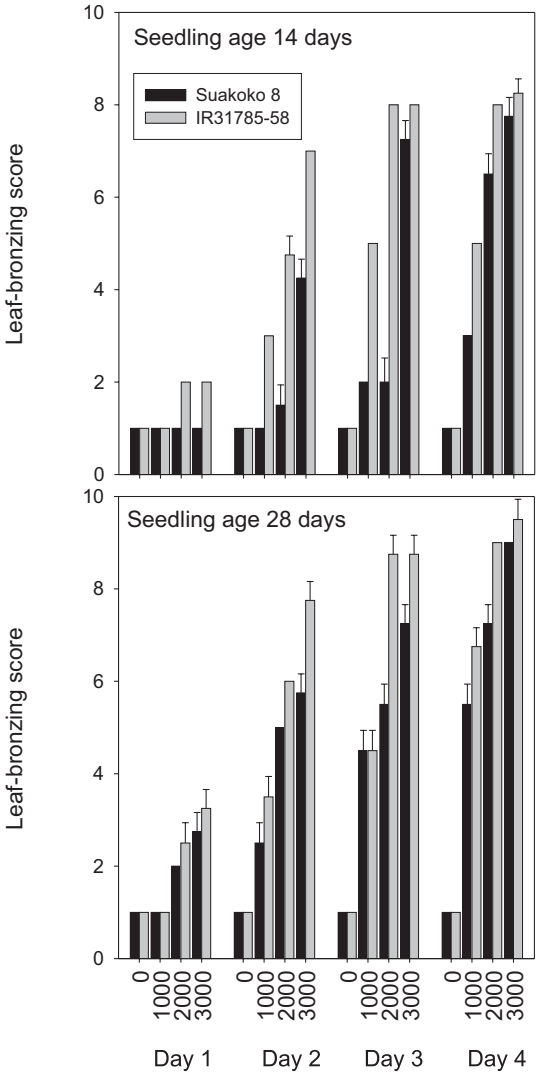

Figure 2: Leaf-bronzing scores for seedlings of two lowland-rice cultivars subjected to different concentrations of Fe in the nutrient solution at two different growth stages. The same seedlings were scored for 4 consecutive days after the addition of Fe to the solution. Error bars = standard error of mean (n = 3). When no error bar is visible, the error was smaller than the resolution of the axis.
Abbildung 2: „Leaf-bronzing"-Boniturwerte für Setzlinge zweier Nassreisgenotypen, die mit unterschiedlichen Konzentrationen an Fe in zwei verschiedenen Wachststumsstadien behandelt wurden. Derselbe Setzling wurde an 4 aufeinanderfolgenden Tagen nach der Zugabe der Fe-Lösung bonitiert. Fehlerbalken = Standardfehler des Mittelwertes (n = 3). Wenn keine Balken zu sehen sind, war der Fehler kleiner als die Auflösung der Achse.

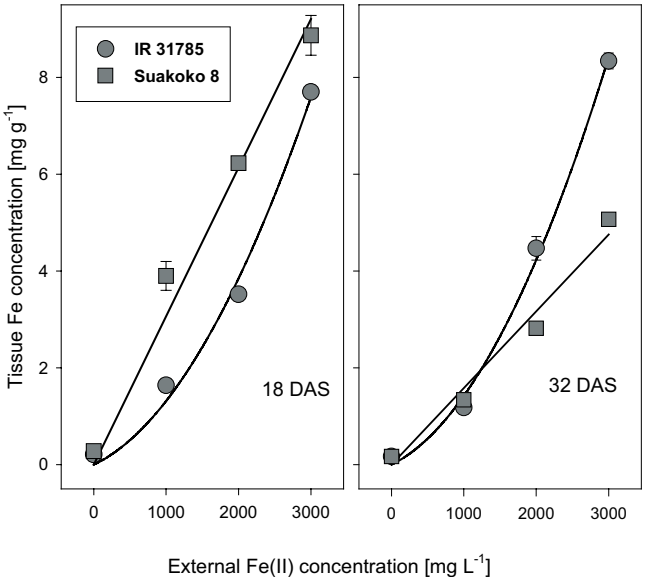

Figure 3: Iron concentration in aboveground plant tissue of two lowland-rice cultivars in relation to external Fe concentrations after 4 d of exposure to different levels of iron stress. Seedlings were grown for either 14 or 28 d before Fe(II) was added to the nutrient solution. All seedlings were sampled after 4 d of exposure. DAS = days after sowing. Error bars = standard error of mean (n = 3). When no error bar is visible, the error was smaller then the symbol size. IR 31785 = IR 31785-58-1-2-3-3.

Abbildung 3: Eisenkonzentrationen der oberirdischen Sprossteile von drei Nassreissorten in Beziehung zur Fe-Konzentration im Nährmedium. Die Setzlinge wurden 14 bzw. 28 Tage angezogen, bevor die unterschiedlichen Fe-Konzentrationen zugesetzt wurden. Alle Setzlinge wurden nach vier Stresstagen geerntet. DAS = Tage nach Aussaat. Fehlerbalken = Standardfehler des Mittelwertes (n = 3). Wenn keine Balken zu sehen sind, war der Fehler kleiner als die Symbolgröße.

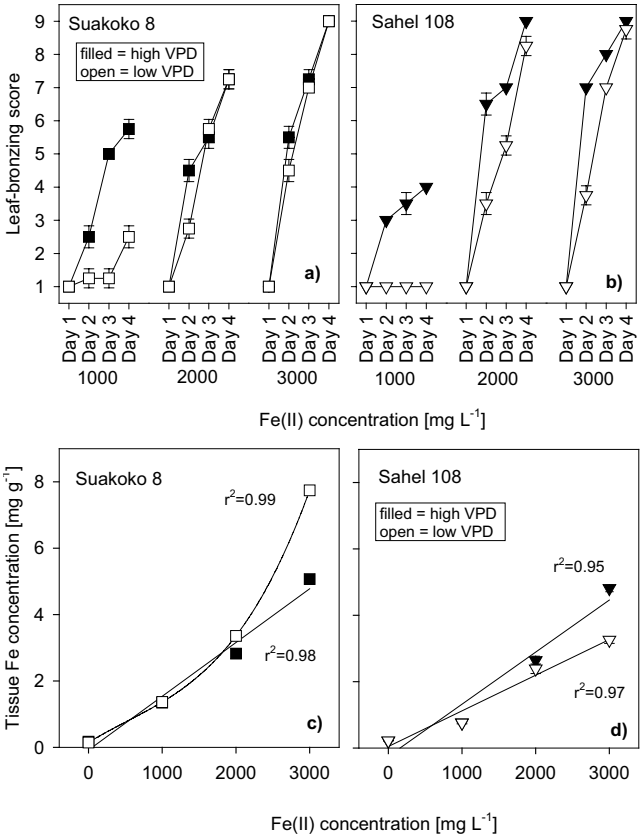

Figure 4: Leaf-bronzing scores (a, b) and tissue Fe concentration (c, d) for seedlings of two lowland-rice cultivars subjected to varying levels of Fe(II) in the nutrient solution and two levels of vapor-pressure deficit (VPD). Plants were treated 28 d after sowing, scored for 4 consecutive days for leaf-bronzing symptoms and samples on day 4 for tissue Fe analyses. Error bars = standard error of mean (n = 3). When no error bar is visible, the error was smaller than the symbol size.

Abbildung 4: „Leaf-bronzing"-Boniturwerte (a, b) und Fe-Gehalte im Gewebe (c, d) zweier Nassreissgenotypen nach Behandlung mit unterschiedlichen Fe-Konzentrationen in der Nährlösung und zwei verschiedenen atmosphärischen Dampfdruckdefiziten. Die Pflanzen wurden 28 Tage nach Aussaat behandelt, an 4 aufeinanderfolgenden Tagen bonitiert und am 4. Tag zur Analyse des Fe-Gehaltes geerntet. Fehlerbalken = Standardfehler des Mittelwertes (n = 3). Wenn keine Balken zu sehen sind, war der Fehler kleiner als die Symbolgröße.

Shoot Fe concentration increased linearly with increasing external Fe(II) in Suakoko8 and exponentially in IR31785-58-1-2-3-3 for both sampling dates (Fig. 3). In young seedlings, Fe concentrations increased faster and to higher levels in tolerant Suakoko8 than in sensitive IR31785-58-1-2-3-3, whereas the opposite was observed in older seedlings. In IR31785-58-1-2-3-3, shoot Fe concentrations ranged from 0.2 to 7.7 mg g^{-1} in 2-week-old seedlings, whereas in Suakoko8, they ranged from 0.28 to 8.8 mg g^{-1}. In the 4-week-old seedlings, shoot-biomass Fe concentrations ranged from 0.17 to 5.1 mg g^{-1} in Suakoko8 and from 0.17 to 8.3 mg g^{-1} in IR31785-58-1-2-3-3.

3.2 Effects of vapor-pressure deficit on leaf symptoms and Fe uptake

Vapor-pressure deficit influences transpiration rates and thus acropetal xylem transport of ions. A decrease in VPD of about 40% is likely to slow down the expression of Fe-toxicity symptoms in leaves. Figure 3 exemplarily shows leaf-bronzing scores (a, b) and leaf-tissue Fe concentration (c, d) for 32 d old Suakoko8 and Sahel108 seedlings subjected to various of Fe concentrations under two different VPD. Low VPD significantly reduced the Fe-toxicity symptoms at an external concentration of 1000 mg L^{-1} Fe^{2+} in both genotypes. At higher concentrations, the influence of VPD on the expression of symptoms was less pronounced in Suakoko8, but significantly delayed the expression of toxicity symptoms in leaves of Sahel108 (Fig. 4a, b). At external concentrations of 2000 and 3000 mg L^{-1} Fe, leaf-bronzing score of Sahel108 on day 2 was thus about 4 at low VPD but about 7 at high VPD. At day 4, maximum leaf-bronzing scores were reached in all treatments and genotypes. Shoot Fe concentrations differed little among VPD treatments within a genotype up to external concentrations of 2000 mg L^{-1}. For the 3000 mg L^{-1} Fe treatment, differing VPD resulted in opposite responses in the two genotypes. In Suakoko8, shoot Fe concentrations were significantly ($p < 0.05$) increased under low VPD, whereas in Sahel108, low VPD significantly reduced shoot Fe concentrations (Fig. 4 c, d).

3.3 Validation of the screening set-up

We observed the largest differentiation between tolerant and sensitive genotypes at external Fe concentrations of 2000 mg L^{-1}, after 3 d of exposure and under conditions of high VPD (Fig. 2). Based on this observation, 14 genotypes were subjected to those conditions at 14 and 28 d after sowing with the aim of validating above findings and to distinguish sensitive and tolerant genotypes. All genotypes included had been tested for Fe responses under field conditions prior to this experiment (Tab. 1). Exposure to Fe stress had no significant effect on seedling dry weight, regardless of the genotype and time of Fe application. Without exposure to Fe, seedling dry weight varied between 6 and 11 mg at 14 DAS and between 29 and 50 mg at 28 DAS (data not shown). No differences in seedling development were observed. Figure 5 shows the cumulative leaf-bronzing score for the 3 d exposure to Fe(II) for all genotypes and the two treatment application dates.

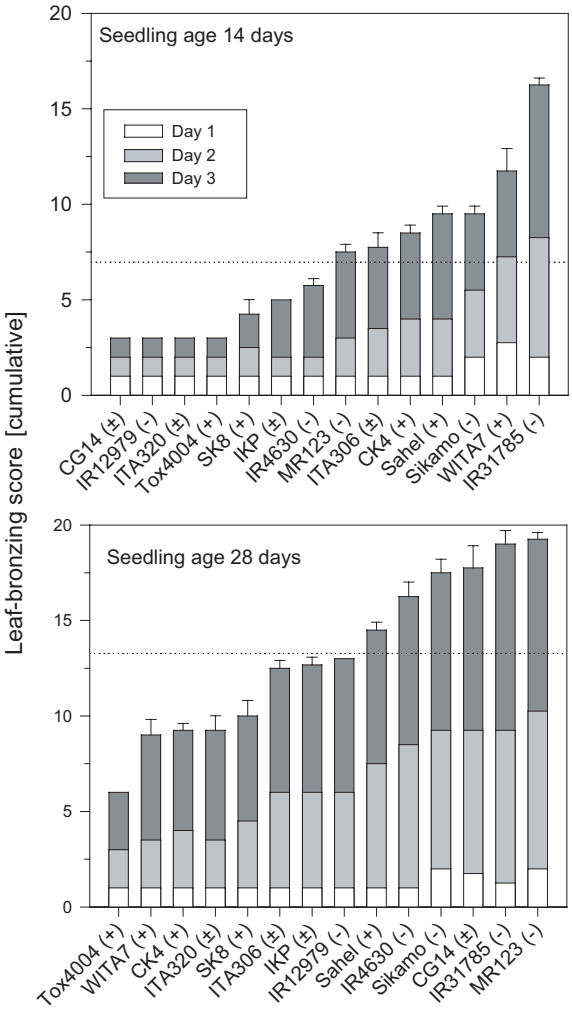

Figure 5: Cumulative mean leaf-bronzing scores for 14 lowland-rice genotypes (Tab. 1). Seedlings were subjected to 2000 mg L^{-1} Fe in the nutrient solution for 3 d under conditions of high VPD either at 14 or at 28 d after sowing. The symbol behind the cultivar name indicates independent estimates of tolerance levels from Tab. 1 (+ = tolerant; ± = intermediate; – = sensitive). Error bars = standard error of mean (n = 3). When no error bars is visible, the error was smaller than the resolution of the axis.

Abbildung 5: Kumulative Darstellung des durchschnittlichen „leaf-bronzing"-Boniturwertes für 14 Nassreisgenotypen (siehe auch Tab. 1). Die Setzlinge wurden entweder 14 oder 28 Tage nach Aussaat über 3 Tage unter hohen atmosphärischen Dampfdruckdefizitbedingungen mit 2000 mg L^{-1} Fe in der Nährlösung behandelt. Das Symbol nach dem Genotypennamen bezeichnet das unabhängig beurteilte Toleranzniveau unter Feldbedingungen (+ = tolerant; ± = moderat empfindlich; – = empfindlich). Fehlerbalken = Standardfehler des Mittelwertes (n = 3). Wenn keine Balken zu sehen sind, war der Fehler kleiner als die Auflösung der Achse.

Symptoms were visible early and 3 d after Fe was applied to the root zone of 2-week-old seedlings, high leaf-bronzing scores were observed in IR31785-58-1-2-3-3, Sahel108, CK4, and WITA7 (Fig. 5, left). On the other hand, Tox4004, ITA320, and CG14 showed barely any symptoms after 3 d of Fe stress. When Fe was applied to 4-week-old seedlings, the distribution of leaf-symptom scores was rather different among the genotypes (Fig. 5, right). Those most susceptible to Fe stress were IR31785-58-1-2-3-3, MR123, IR4630-22-2, and CG14. The genotypes with least symptoms included Tox4004, CK4, Suakoko8, and WITA7. In the sensitive genotypes, symptom expression on the second day of stress had the largest share in the cumulative score, whereas in the resistant genotypes, the major share of symptom expression occurred on day 3.

In general, high tissue Fe concentrations resulted in high leaf-bronzing scores, however, at 14 d after sowing, this correlation was not significant, and the scatter suggests the presence of mechanisms resulting in low bronzing scores while allowing high tissue concentrations of Fe in at least three genotypes. (Fig. 6, left). At 28 d after sowing, a significant correlation between bronzing score and Fe concentration in the shoot was found (Fig 6, right). However, the correlation was only significant with Tox4004 and Suakoko8 being excluded from the regression.

4 Discussion

4.1 Genotype differentiation and seedling age

One of the problems in field screening of large numbers of genotypes for tolerance to Fe-toxic conditions is to provide sufficiently homogenous and elevated Fe levels in the soil, thus providing comparable stress levels to all materials (*Audebert* and *Sahrawat*, 2000). Equally, screening under controlled conditions faces the problem of controlling Fe^{2+} concentrations in hydroponic solutions due to re-oxidation of Fe^{2+} into Fe^{3+} by atmospheric oxygen (*Bode* et al., 1995). Our set-up provided sufficient nutrients for normal seedling growth for 2–4 weeks and allowed to apply a strong pulse of Fe(II) to the root system under anaerobic conditions due to the paraffin layer blocking atmospheric oxygen. Iron pulses of up to five times the concentration used in other studies (*Van Breemen* and *Moorman*, 1978; *Bode* et al., 1995) induced a wide range of leaf-symptom scores in the test genotypes and resulted in significantly different Fe concentrations in the leaf tissue within 48 h (Figs. 1 and 2). Three days exposure to an external concentration of 2000 mg L^{-1} Fe induced signifi-

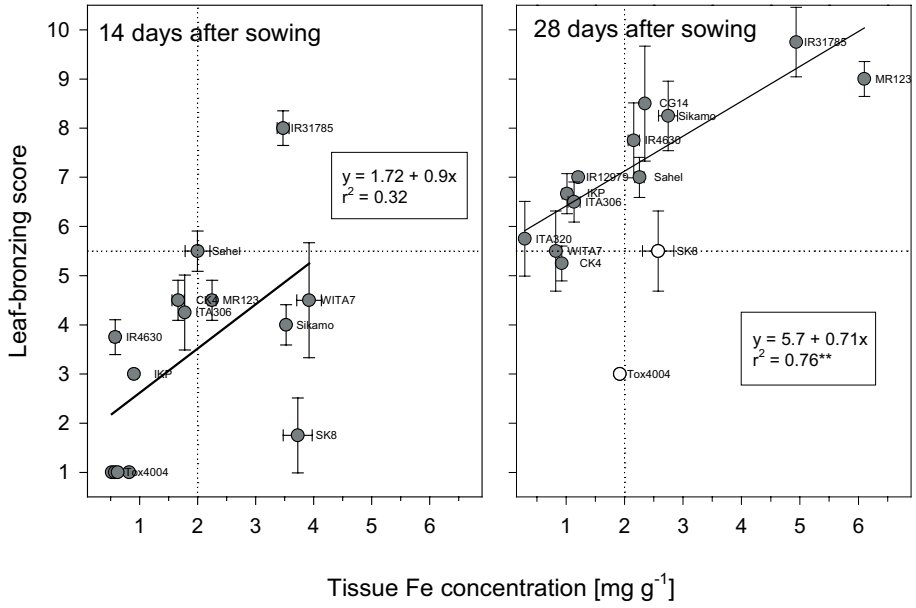

Figure 6: Leaf-bronzing scores as related to tissue Fe concentration for 14 lowland-rice genotypes. For details of the set-up refer to Fig. 4. Genotypes shown with an open symbol on the right were not included in the regression. Error bars = standard error of mean (n = 3). When no error bar is visible, the error was smaller than the symbol size. ** = highly significant ($p < 0.01$).
Abbildung 6: „Leaf-bronzing"-Boniturwerte in Beziehung zu den Fe-Gehalten in den oberirdischen Sprossteilen von 14 Nassreisgenotypen. Details siehe Abb. 4. Genotypen, die mit einem offenen Symbol in der rechten Abbildung dargestellt sind, wurden bei der Regressionsanalyse nicht berücksichtigt. Fehlerbalken = Standardfehler des Mittelwertes (n = 3). Wenn keine Balken zu sehen sind, war der Fehler kleiner als die Symbolgröße. ** = hochsignifikant ($p < 0{,}01$).

cantly different leaf-bronzing scores for tolerant Suakoko8 and sensitive IR31785 in both 14 and 28 d old seedlings. Seedlings subjected to high concentrations of external Fe^{2+} at 28 DAS developed leaf-bronzing symptoms faster and had higher leaf-bronzing scores than seedlings subjected to the same conditions at 14 DAS (Figs. 1 and 5). This, however, was not reflected in leaf-tissue Fe concentrations (Fig. 2). Sahel108 and IR31785 accumulated approximately the same tissue Fe concentrations at both seedling ages, while scoring significantly differed for leaf-bronzing symptoms at the two growth stages (Figs. 1, 2, and 4). Suakoko8 on the other hand had higher leaf-bronzing scores when subjected to high concentration of external Fe at 28 DAS (Figs. 1 and 4), but leaf-tissue Fe concentrations were significantly lower than at 14 d (Fig. 2 and 5). These data suggest that Fe uptake is regulated by different mechanisms in the different genotypes. Sahel108 seemed to exclude Fe quite efficiently (Fig. 2), but sensitivity to Fe taken up to the leaves increased with plant age as leaf-bronzing scores in older seedlings were higher than in younger ones (Fig. 5), while the leaf-tissue concentration of Fe did not differ (Fig. 2). IR31785 apparently cannot efficiently exclude Fe, and Fe accumulation increased proportionally to external Fe concentrations, causing severe leaf-bronzing symptoms. Suakoko8 accumulated at least twice as much Fe in leaf tissues as IR31785 when subjected to high external Fe concentrations at 14 DAS (Fig. 2) but leaf-bronzing scores were generally lower than in IR31785. When Suakoko8 seedlings were treated at 28 DAS, tissue Fe concentration did not exceed those of Sahel108 (Fig. 2), however, bronzing scores increased faster and to the same final level as in the younger seedlings (Fig. 1).

Under conditions of Fe toxicity, rice plants have two general ways to cope with the Fe stress: (1) avoidance of toxic Fe levels in plant tissues through regulation of Fe uptake and (2) tolerance of elevated Fe concentrations in the leaf tissue. Rice can avoid toxic levels of Fe in the tissue by either oxidizing of Fe^{2+} in the rhizosphere or by discriminating against Fe at the root endodermis. The former is achieved through an oxidation barrier in the rhizosphere established by channeling molecular oxygen from the atmosphere through the stems into the roots *via* a gas-conducting tissue or aerenchyma (*Ando*, 1983). Under anaerobic conditions, aerenchyma formation starts in 2- to 4-week-old plants, and the oxidation power of the root is highest at the maximum tillering stage (*Tadano*, 1975).

Root-cell membranes can discriminate against reduced Fe (*Tadano*, 1976), explaining the deposition of Fe(III) in the apoplast of root parenchymatic cells observed by *Green* and *Etherington* (1977). Xylem-sap analysis of 2-month-old rice plants indicated that up to 87% of the Fe reaching the root apoplast by mass flow was subsequently not detected in the xylem and must hence have been "excluded" at the endodermal barrier between cortex and stele (*Yamanouchi* and *Yoshida*, 1981). There was no such reduction found at the seedling stage, and reduction levels remained low throughout the early growth stages of rice (*Tadano* and *Yoshida*, 1978).

Thus, seedling younger than 4 weeks have in most cases not yet developed effective avoidance mechanisms at root level, explaining differences in tissue Fe concentrations between 2- and 4-week-old seedlings observed in this study.

Quite in contrast to avoidance mechanisms that develop only in advanced vegetative growth stages, severe leaf-bronzing symptoms are commonly observed in older seedlings (*Yamanouchi* and *Yoshida*, 1981). *Prade* (1987) observed high tissue Fe concentrations in young, field-grown rice seedlings, however, Fe-toxicity symptoms only appeared in older plants, the tissue Fe concentrations of which were reduced to less than half of the initial one. Free radicals are responsible for the tissue damage caused by Fe toxicity (*Thongbai* and *Goodman*, 2000). They irreversibly damaged membrane lipids (*Thompson* and *Legge*, 1987), proteins (*Chevrier* et al., 1988; *Miyao* et al., 1995), and nucleic acids (*Elstner*, 1982). The activity of phenol oxidases increases, and oxidized polyphenols accumulate causing leaf-bronzing symptoms (*Peng* and *Yamauchi*, 1993). In the present study, tissue Fe concentrations in some cases were lower in 4-week-old seedlings than in 2-week-old seedlings, nevertheless, leaf-bronzing symptoms were either as severe or even more severe in the older seedling than in the younger ones. Since young plants in general have lower polyphenol concentrations in the leaves (*Sharma* and *Vaid*, 1997; *Cartelat* et al., 2004), it is likely that leaf-bronzing symptoms caused by the activity of phenol oxidases did not occur in young seedlings, since phenol levels were too low. Thus, the apparently higher sensitivity of older leaves to elevated tissue Fe concentrations as observed by *Yamanouchi* and *Yoshida* (1981) was probably due to increased concentrations of polyphenols in older leaves.

Concluding the discussion above, we can say that sensitive and tolerant genotypes could be clearly differentiated by the method used. The differences between the genotypes were most obvious at 2000 mg L^{-1} Fe in the external nutrient solution and after 3 d of exposure. Changes in the relationship between leaf-bronzing symptoms and tissue Fe concentration between 2- and 4-week-old seedlings suggest that 2-week-old seedlings may not be sufficiently mature to have developed adequate response mechanisms to Fe toxicity.

4.2 Effects of vapor-pressure deficit

Abiotic-stress effects are often more severe under conditions of high VPD as compared to low VDP (*e.g.*, for drought *Asch* et al., 2003a, and for salinity *Asch* and *Wopereis*, 2001). Iron toxicity occurs in a wide range of agro-ecological zones from the humid forest zone to semiarid regions. Thus, it is to be expected, that the effects of Fe toxicity on a given genotype differ among system and seasons. *Sahrawat* and *Singh* (1998) have shown that Fe-toxicity effects in lowland-rice cultivars differ between dry- and wet-season crops. In the present study, VPD in the growth chamber was relatively high (1.75 kPa). To evaluate the influence of air humidity on the uptake of Fe and the development of bronzing symptoms, we decreased VPD to about 60% of the ambient conditions. Particularly at low external Fe concentrations, lower VPD had a mitigating influence on the expression of leaf-bronzing symptoms (Fig. 3). This influence became less important as the external Fe concentration increased. Final bronzing scores were always very similar. At high external Fe concentration, Fe-uptake rates and Fe content in the canopy increases proportionally to the amount of water lost to the atmosphere (*Tadano* and *Yoshida*, 1978). Thus, under conditions of high transpiration (*i.e.*, during the exponential-growth phase of rice or under dry-season conditions when crop transpiration rates are enhanced [*Asch* et al., 2003b]), the rate of acropetal Fe(II) transport into the leaves is likely to increase. Increased tissue Fe concentrations can lead to an accumulation of oxidized polyphenols causing leaf-bronzing symptoms due to increased activity of phenol oxidases which in turn is stimulated by increasing water deficit (*Thipyapong* et al., 2004). The VPD conditions chosen in this study were well suited to differentiate between genotypes as sensitive genotypes developed leaf symptoms faster, while tolerant genotypes were not significantly influenced by VPD.

4.3 Validation and mechanistic screening

Fourteen genotypes were subjected to the conditions defined in the first two parts of this study. When seedlings were subjected to Fe toxicity at 14 DAS, the tested germplasm segregated based on leaf-bronzing symptoms into seven tolerant, five intermediate, and two sensitive genotypes. The same germplasm subjected to Fe toxicity 2 weeks later segregated into five tolerant, four intermediate, and five sensitive genotypes (Fig. 4). However, the composition of the groups was rather different. The changes in bronzing score due to differences in growth stage support the conclusion that scoring the very young seedlings does not provide a reliable screen. Among the 4-week-old seedlings, Suakoko8, CK4, Tox4004, and WITA7 confirmed Fe-toxicity scores determined in field trials (*WARDA*, 1995, 1998).

One of the objectives of this study was to investigate the possibility of deriving information about adaptation mechanisms to excess Fe from such a simple screen. Thus, we determined the tissue Fe concentration for all genotypes and plotted that against the leaf-bronzing score (Fig. 5). This clearly revealed that younger seedlings generally have less bronzing symptoms but higher tissue Fe concentrations than older seedlings. Exceptions were those four genotypes (Fig. 5a) that apparently did not take up any Fe at 14 DAS. This is probably due to a not yet fully developed metabolism and thus very low transpiration and assimilation rates as shown by *Asch* et al. (1999). In older seedlings, leaf-bronzing score and tissue Fe concentrations were highly significantly correlated ($p < 0.01$, Fig. 5). This allowed distinguishing between sensitive includers (*e.g.*, IR31785 and MR123) and resistant excluders (*e.g.*, WITA7 and CK4) with the exclusion mechanisms being either oxidation power of the root (well established aerenchyma) or symplastic discrimination. Scoring the leaf bronzing after 3 d of exposure to 2000 mg L^{-1} Fe^{2+} under conditions of high VPD permitted to identify genotypes resistant to Fe toxicity. Combining the leaf-bronzing score of those genotypes with the tissue Fe concentration revealed the different resistance strategies. While ITA320, WITA7, and CK4 apparently efficiently excluded Fe^{2+}, Suakoko8 showed a similar bronzing score but at a three times higher tissue Fe concentration, therefore obviously tolerating elevated levels of Fe in the tissue. Tox4004 had intermediate tissue Fe concentration with only about half the bronzing score of the genotypes described above, suggesting a combination of exclusion and tolerance mechanisms.

5 Conclusions

We have shown that with this simple and relatively cheap screening set-up, genotype responses to Fe toxicity can be differentiated as early as 28 d after sowing. Controlled conditions allow screening under identical conditions and thus eliminate the uncertainties of field screening. Cascading leaf-bronzing score as the initial screen with tissue-Fe analyses to separate resistance mechanisms proved effective to identify differences in adaptation strategies within the genotype spectrum. Further research is needed to tie the different mechanisms to inheritable traits. We are in the process of developing this tool further and to screen segregating populations containing microsatellite markers to identify quantitative trait loci (QTLs) associated with different adaptation strategies of rice to conditions of Fe toxicity.

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