Research Article

Diversity in the genome of *Aegilops tauschii*, a wild wheat relative, to generate Fe-biofortified and Fe-deficiency-tolerant wheat

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Abstract

Iron (Fe) is an essential element for all organisms. Fe deficiency can limit plant production and cause anaemia in humans. The improvement of Fe homoeostasis could resolve both problems. Fe homoeostasis in *Aegilops tauschii*, the D genome donor of bread wheat, is not fully understood. Here, we analysed physiological traits in 42 accessions of *Ae. tauschii* associated with Fe homoeostasis, i.e. mugineic acid family phytosiderophores (MAs), phenylamides, SPAD values and metal concentrations. All traits showed diversity, suggesting the presence of candidate genes in the *Ae. tauschii* accessions, which could improve Fe homoeostasis in bread wheat. All accessions mainly produced and secreted mainly 2′-deoxymugineic acid among MAs, but eight of them secreted unknown products from their roots under Fe deficiency. It was revealed that 15 kinds of phenylamides and 2 kinds of bread wheat phytoalexins were produced in Fe-deficient roots of *Ae. tauschii*. Multivariate and principal component analyses showed that chlorophyll content was correlated with shoot Fe concentration. Genome-wide association study analysis associated several genomic markers with the variations in each trait analysed. Our results suggest that *Ae. tauschii* has alleles that could improve Fe homoeostasis to generate Fe-deficiency-tolerant or Fe-biofortified bread wheat.

Introduction

Iron (Fe) is an essential micronutrient for all living organisms. Fe plays a key role in electron transfer in both photosynthetic and respiratory reactions. In plants, Fe deficiency impairs chlorophyll synthesis and causes leaf yellowing due to Fe chlorosis, leading to low yield and poor nutritional quality (Marschner *et al.*, 1986). In humans, Fe deficiency causes anaemia, which is associated with several comorbidities, including cardiovascular disease and is an independent risk factor for death (Groenveld *et al.*, 2008; Parikh *et al.*, 2011; Klip *et al.*, 2013). Humans take in Fe, which is principally taken up by plants from the soil. Indeed, a decrease in the Fe concentrations in food was correlated with increasing Fe deficiency anaemia (Sun and Weaver, 2021). Therefore, increasing Fe uptake efficiency could contribute both to increasing plant productivity and improving human health.

Fe is abundant in the soil, but most of it is not available to plants, being oxidized to Fe(III) hydroxide ($Fe(OH)_3$), which is poorly soluble in water. This phenomenon is exacerbated in high-pH soils, including calcareous soils, which account for an estimated 30% of the world's cultivated land, in which plants often exhibit severe chlorosis due to Fe deficiency constituting a major agricultural problem for crop production (Marschner, 1995; Mori, 1999). Inappropriate irrigation in drylands leads to the accumulation of salts, increasing soil pH (FAO, 2022). Irrigated salt-affected soils were estimated as covering 20 to 50% of all irrigated land (FAO, 2022). In these regions, Fe deficiency is a common agronomic problem. Thus, Fe-deficiency-tolerant plants might stay green on calcareous soils.

Organisms have evolved methods to take up insoluble Fe(III). Grasses, including important staple crops such as wheat, rice and barley, secrete natural Fe chelators called mugineic acid family phytosiderophores (MAs) from their roots (Takagi, 1976) through the Transporter Of MAs (TOM1) (Nozoye *et al.*, 2011). MAs chelate and solubilize $Fe(OH)_3$ in the soil, forming Fe(III)–MAs complexes, which are taken into root cells via Fe(III)–MAs transporters called yellow stripe 1 (YS1)/YS1-like (YSL) transporters, localized to the root cell membrane (Curie *et al.*, 2001; Inoue *et al.*, 2009). MAs are suggested to play an important additional role in the long-distance transport of metals (Mori *et al.*, 1991; Kawai *et al.*, 2001; Kakei *et al.*, 2009; Nishiyama *et al.*, 2012; Nozoye *et al.*, 2015) and can chelate not only Fe but also various

transition metals such as zinc (Zn), manganese (Mn) and copper (Cu) (Murakami *et al.*, 1989; Mori *et al.*, 1991), being involved in metal homoeostasis, which is important for plant development.

The production and secretion of MAs increase markedly in response to Fe deficiency. Although wheat, rice and maize secrete 2′-deoxymugineic acid (DMA), other species, including barley and rye, hydroxylate DMA to other MAs. Takagi *et al.* (1984) suggested that the kinds and amounts of MAs are positively correlated with plant tolerance to Fe deficiency. Indeed, transgenic enhancement of the production and secretion of MAs and increase of the kinds of MAs conferred Fe-deficiency-tolerance in plants (Takahashi *et al.*, 2001; Suzuki *et al.*, 2008; Ogo *et al.*, 2011), suggesting that the abilities of MAs production and secretion are key factors in plant tolerance of Fe deficiency. We expect that the ability to make new MAs would further promote tolerance.

Other than MAs, plants also respond to Fe deficiency by enhancing root secretion of phenolic compounds (Römheld and Marschner, 1983; Susín *et al.*, 1993; Rodríguez-Celma *et al.*, 2011; Donnini *et al.*, 2012). Phenolics are considered putative external Fe reductants or chelators (Brown and Ambler, 1973). In grasses, it is suggested that phenolics such as protocatechuic acid solubilize and chelate Fe(III) and then reduce it and translocate the precipitated insoluble Fe(III) in the plant body (Bashir *et al.*, 2011; Ishimaru *et al.*, 2011). In dicots, phenolics such as coumarins, which are produced via the phenylpropanoid pathway, are secreted from the roots and are involved in Fe uptake from the rhizosphere (Rodríguez-Celma *et al.*, 2013; Schmid *et al.*, 2013; Fourcroy *et al.*, 2014; Schmidt *et al.*, 2014), in addition to its translocation in the plant body (Tsai *et al.*, 2018). An extensive study of plant diversity could find candidate genes that can improve Fe use efficiency.

Bread wheat (*Triticum aestivum* L.) is an important crop for global food security. It is grown on a greater area than any other crop and provides one-fifth of all calories consumed by humans (Simons *et al.*, 2014). Bread wheat originated through few events of natural hybridization between durum wheat (*T. turgidum* L. subsp. *durum*) and *Aegilops tauschii* Coss. (syn. *Ae. squarrosa* auct. non L.). Because these events involved few progenitors, the genetic diversity of durum wheat and *Ae. tauschii* is not fully represented in current bread wheat germplasm (Gill and Raupp, 1987; Dreisigacker *et al.*, 2008; Li *et al.*, 2014). By introducing the genes of wild species, it might be possible to generate bread wheat which can take up more Fe from soils and which could grow under Fe-deficient conditions such as calcareous soils and drylands. Such genes could also be used to generate biofortified bread wheat with a high Fe content.

Ae. tauschii is a wild diploid self-pollinating species ($2n = 2x = 14$, DD) and was the donor of the D genome to the hexaploid bread wheat ($2n = 6x = 42$, AABBDD) (Kihara, 1944; McFadden and Sears, 1946). Molecular phylogenetic analysis divided *Ae. tauschii* into three groups in two main lineages (TauL1 and TauL2) and one minor lineage (TauL3), suggesting diversity among *Ae. tauschii* accessions (Matsuoka *et al.*, 2013; Mahjoob *et al.*, 2021). Since wild *Ae. tauschii* is widespread, it is speculated that it holds many useful genes that can improve the yield and quality of bread wheat (Ogbonnaya *et al.*, 2013). Indeed, physiological traits such as heading date and mineral contents ranged widely in a population of synthetic bread wheat lines harbouring genomic fragments of *Ae. tauschii* (Gorafi *et al.*, 2018a, 2018b). Thus, physiological and genetic analyses of *Ae. tauschii* accessions might find new contributors to the efficient uptake and translocation of Fe.

Here, we report the comprehensive physiological analysis of Fe homoeostasis in *Ae. tauschii*, revealing genetic diversity that can be used to generate Fe-deficiency-tolerant or Fe-biofortified bread wheat.

Materials and methods

Plant materials and analysis of plant physiological traits

We used 42 accessions of *Ae. tauschii* that have been used to generate synthetic wheat lines (Gorafi *et al.*, 2018a), the tetraploid *T. turgidum* subsp. *durum* 'Langdon' and the hexaploid *T. aestivum* 'Norin 61'. All plants were grown hydroponically in a growth chamber under a 14-h light / 10-h dark regimen at 20°C. The seeds were germinated on paper wetted with tap water for 7–12 days; then, they were moved to a 20-l plastic container containing the nutrient solution of the following composition: 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.5 µM $ZnSO_4$, 0.05 µM Na_2MoO_4 and 0.1 mM Fe-EDTA described by Nozoye *et al.* (2011). Fe deficiency was initiated 4–5 weeks after germination by transfer of the plants to an Fe-free culture medium. After 7 days, the root exudates were collected as described in the next section and plants were harvested to analyse chlorophyll (SPAD), phenylamides, phytoalexins and metals. SPAD values of the three most expanded leaves were measured with a SPAD-502 chlorophyll meter (Konica-Minolta, http://www.konicaminolta.com) and averaged. Dried shoots were ground and used for metal determination by inductively coupled plasma atomic emission spectrometry as described (Nozoye *et al.*, 2017). All experiments were performed in triplicate.

Collection of root exudates

MAs secretion was measured as described (Nozoye *et al.*, 2014). In brief, roots were rinsed with deionized water and then held in deionized water for 5 h from the start of illumination. The water was renewed once and an antimicrobial agent (Micropur; Katadyn Products Inc.) was added to prevent microbial degradation of the MAs. These root exudates were filtered through Amberlite IR(H⁺) 120 (Organo) cation exchange resin and the cationic fraction eluted with 2 M NH_4OH was analysed by high-performance liquid chromatography (HPLC) as described (Nozoye *et al.*, 2011).

Analyses of phenylamides in root extracts

Phenylamides were prepared as described (Morimoto *et al.*, 2018; Ube *et al.*, 2019a, 2019b). Plant materials were extracted in 10 vol. of 80% methanol for 24 h, and the extracts were subjected to liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis with multiple reaction monitoring on a triple-quadrupole LC-MS/MS system (Agilent 6420, Agilent) connected to an ultra-HPLC system (1290 Infinity II, Agilent), with the following LC conditions: column, Acquity UPLC BEH C18, 2.1 mm × 50 mm (1.7 µm; Waters); flow rate, 0.2 ml/min; column temperature, 40°C; solvents, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); gradient, 5–70% A/(A + B) within 10 min. The multiple reaction monitoring conditions were optimized using authentic compounds (online Supplementary Table S1).

We analysed 25 phenylamides – all 25 combinations of 5 acids (cinnamic acid, Cin; caffeic acid, Caf; *p*-coumaric acid, Cou;

ferulic acid, Fer; and benzoic acid, Ben) and 5 amines (putrescine, Put; agmatine, Agm; tryptamine, Try; serotonin, Ser; and tyramine, Tyr) – and 2 phenylamide phytoalexins, triticamide (Tri) A (cinnamoyl-9-hydroxy-8-oxotryptamine) and TriB (cinnamoyl-8-oxotryptamine). We refer to each phenylamide by combining the abbreviations of their acid and amine parts, e.g. CouPut = *p*-coumaroylputrescine.

Genome-wide association study (GWAS), gene identification and statistical analysis

We performed GWAS as described (Elhadi *et al.*, 2021) for DMA secretion from roots, leaf SPAD values, metal (Fe, Zn, Mn and Cu) concentrations in shoots and the six phenylamides and two phytoalexins abundant in the roots of *Ae. tauschii*. Best linear unbiased prediction (BLUP) values for each trait and a Mixed Linear Model (MLM) in TASSEL v. 5 software were used. To identify candidate genes, we used significant marker sequences for searches in Gramene (https://www.gramene.org/) with the BLAST option for comparison with the *Ae. tauschii* sequence (Aet_v4.0). To find the annotations of each marker, we performed BLAST analysis using the marker sequence as a query against the *Ae. tauschii* genome data set (Aet_v4.0) in Gramene and National Center for Biotechnology information (NCBI; National Center for Biotechnology Information (nih.gov)). When the sequence of a marker did not overlap a gene, the closest gene was retrieved. Multivariate analysis and principal component analysis (PCA) were performed in JMP Pro v. 16 software (SAS Institute Inc., https://www.jmp.com/en_us/home.html).

Results

Analysis of MAs in root exudates of Ae. tauschii

The main peaks detected in all root exudate samples were consistent with DMA (Fig. 1a). Small peaks were detected before the peak of DMA in accessions KU-2109, IG126387, KU-2075, KU-2092, KU-2093, KU-2096, KU-2097 and KU-2156, but these peaks differed from mugineic acid (MA) and 3′-hydroxymugineic acid (HMA). In KU-2093, KU-2097, KU-2156, there was a peak between DMA and asparagine (ASP). The variation in the peaks seemed not to be linked to lineage (TauL1, 2, or 3). DMA concentration was estimated at 0.004 to 56.9 µg/g FW per 5 h (Fig. 1b).

Analysis of phenolics in roots of Ae. tauschii

In the roots of *Ae. tauschii* and wheat plants, we detected 15 phenylamides – CafPut, CouPut, FerPut, CinAgm, CouAgm, FerAgm, BenAgm, CafAgm, BenTry, CinTry, CouTry, FerTry, FerSer, CouTyr and FerTyr – and both phytoalexins (TriA and TriB) (Fig. 2). Among them, CouPut, FerPut, CouAgm and FerAgm were abundant, though FerPut and FerAgm were variable, and CafPut, CinAgm, TriA and TriB were low and highly variable. Differences among TauL1, TauL2 and TauL3 were not clear.

Analysis of SPAD values and metal concentrations in shoots of Ae. tauschii

The SPAD values and metal concentrations varied among the lines of *Ae. tauschii*, although there were no clear differences among TauL1, TauL2 and TauL3 (Fig. 3).

Multivariate analysis and PCA

SPAD values were correlated positively with Fe concentrations in the shoots and negatively with DMA secreted from the roots (Fig. 4a). They were correlated slightly positively with Cu, Zn, Mn, CouPut, CinAgm, CouAgm and FerAgm and slightly negatively with CafPut, FerPut, TriA and TriB.

Fe concentrations in the shoots were correlated positively with Cu, Mn, Zn, CouPut, FerPut and CouAgm, in addition to SPAD value. Fe was correlated more strongly with Cu than with Mn and Zn. Cu, Mn and Zn were correlated strongly positively with each other and moderately positively with CouPut.

There were several positive correlations among phenylamides and phytoalexins. CafPut was correlated strongly with CinAgm and weakly with CouPut, CouAgm, TriA and TriB. CouPut was correlated strongly with CouAgm and CinAgm. FerPut was correlated strongly with CouAgm and FerAgm. CinAgm was correlated strongly with CafPut, CouPut, CouAgm, TriA and TriB. TriA and TriB were correlated with each other and with DMA secreted from the roots.

PCA separated the data into two main groups, one with SPAD values and Fe, Zn, Mn and Cu in the shoots and the other with phenylamides and phytoalexins (Fig. 4b). CouPut and DMA did not belong to either group.

GWAS analysis

Manhattan plots showed that some markers were correlated with each physiological trait (Fig. 5; Table 1, online Supplementary Table S2). Among the markers most closely associated with each trait, those associated with Zn, DMA, CafPut, CinAgm, TriA and TriB overlapped a gene coding region (Table 1). For other traits, overlapping genes were not found, so the closest genes are listed. Among the gene extracted, some traits shared the same genes (online Supplementary Table S2). Among those associated with Cu, Mn and Zn, five were the same as each other. In addition, among 10 genes associated with Cu, 2 were also associated with Zn and 1 was also associated with Cu. Such duplications were also found between FerAgm and FerPut, CinAgm and TriB and TriA and TriB.

Discussion

New MAs in Ae. tauschii might be useful to enhance Fe homoeostasis in bread wheat

Both the amounts of MAs secreted from roots and the number of MAs types are positively correlated with tolerance to Fe deficiency (Takagi *et al.*, 1984). MAs are produced from *S*-adenosylmethionine to DMA, the first MAs, via four sequential steps catalysed by *S*-adenosylmethionine synthetase, nicotianamine synthase, nicotianamine aminotransferase and DMA synthase. (Mori and Nishizawa, 1987; Shojima *et al.*, 1990; Higuchi *et al.*, 1999; Takahashi *et al.*, 1999; Bashir *et al.*, 2006). As in wheat, rice produces and secretes only DMA. Introducing the barley Fe-deficiency-specific clone no. 3 gene (*IDS3*) (Nakanishi *et al.*, 2000) for the hydroxylase which converts DMA to MA conferred tolerance to Fe deficiency in calcareous soil (Suzuki *et al.*, 2008). Wheat with a rye genome added to it became able to produce HMA and MA in addition to DMA (Mori *et al.*, 1990). Although the D genome is already present in cultivated wheat, the intraspecific diversity of the D genome is in wild *Ae. tauschii* accessions may carry novel and useful genes for involvement in
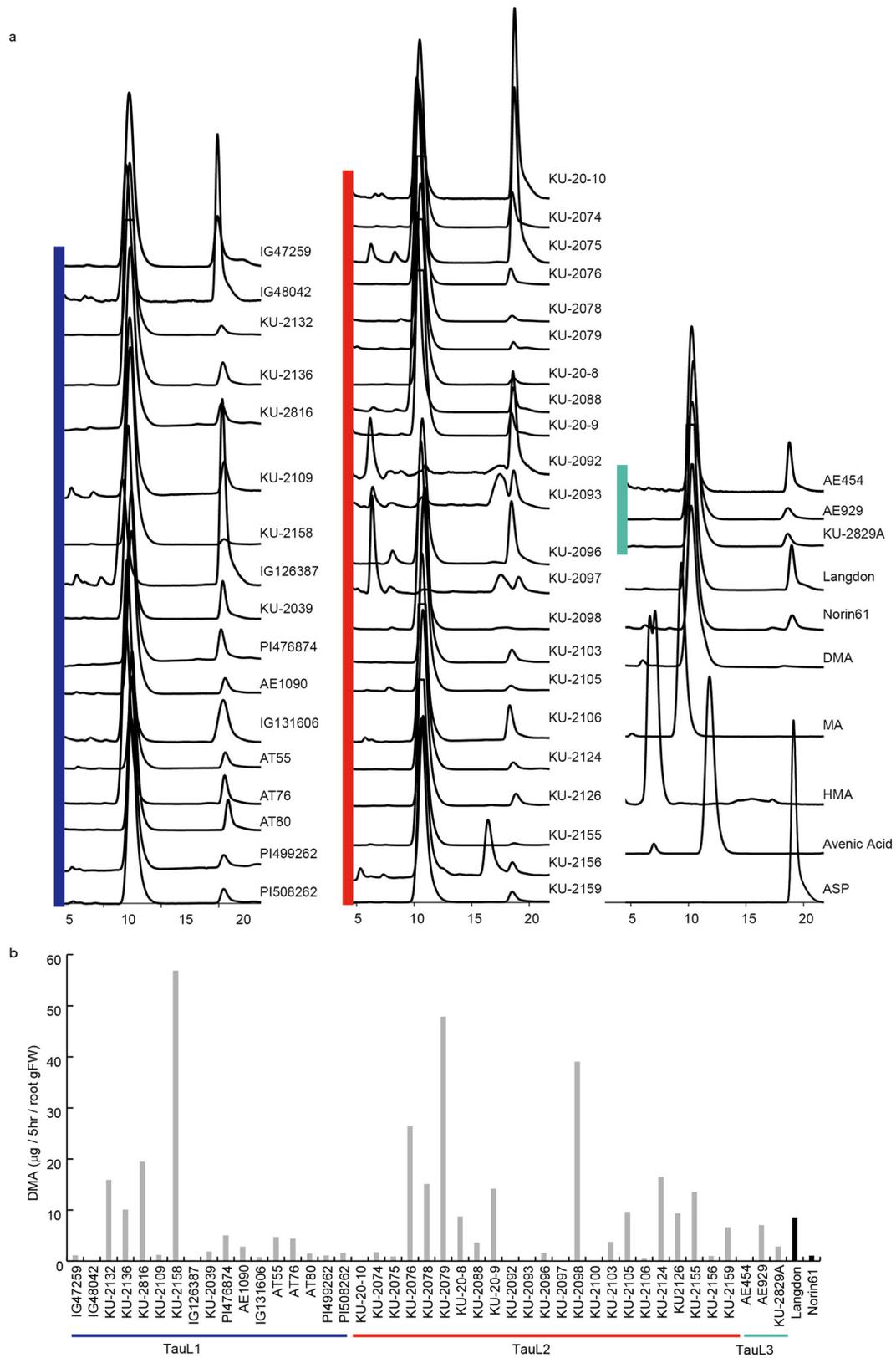
Figure 1. HPLC analysis of *Ae. tauschii* root exudates. (a) Chromatograms of root exudates of 42 *Ae. tauschii* lines and wheat 'Norin 61' and 'Langdon'. Standard solutions of 2′-deoxymugineic acid (DMA), mugineic acid (MA), 3′-hydroxymugineic acid (HMA), avenic acid and aspartate (ASP) were used as controls. The *x*-axis represents the retention time (min). Retention times: DMA standard, 12.48 min; MA, 10.08 min; HMA, 7.95 min; avenic acid, 11.63 min; ASP, 19.13 min. (b) DMA secretion from roots calculated from the peak area of DMA of each accession. Lineages by molecular phylogenetic analysis are shown in colour: blue, TauL1; red, TauL2; teal, TauL3.
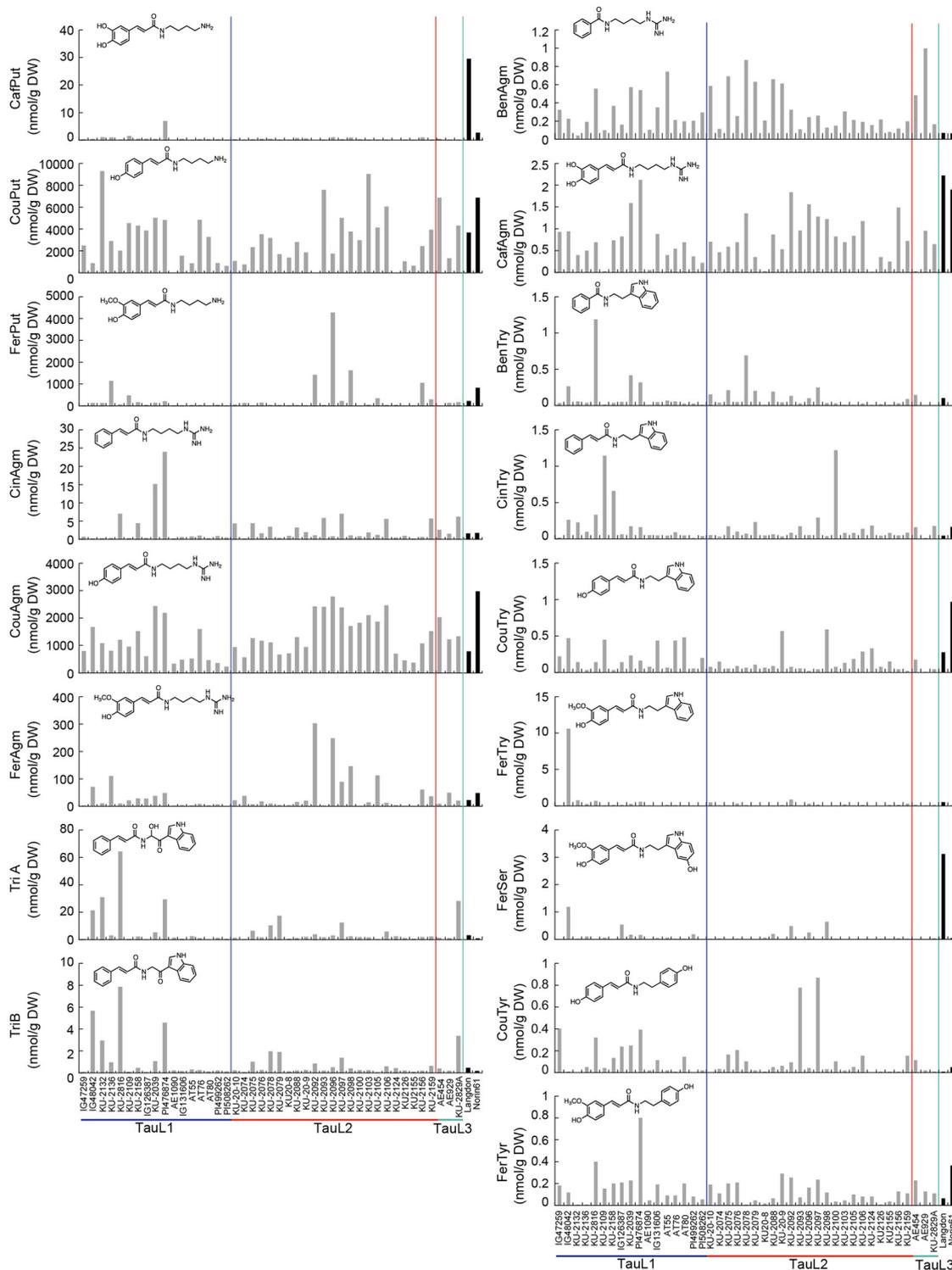
Figure 2. Analysis of phenylamides in roots of *Ae. tauschii* accessions and wheat 'Norin 61' and 'Langdon'. LC-MS/MS detected 15 phenylamides – caffeoylputrescine (CafPut), *p*-coumaroylputrescine (CouPut), feruloylputrescine (FerPut), cinnamoylagmatine (CinAgm), *p*-coumaroylagmatine (CouAgm), feruloylagmatine (FerAgm), benzoylagmatine (BenAgm), caffeoylagmatine (CafAgm), benzoyltryptamine (BenTry), cinnamoyltryptamine (CinTry), *p*-coumaroyltryptamine (CouTry), feruloyltryptamine (FerTry), feruloylserotonin (FerSer), *p*-coumaroyltyramine (CouTyr), feruloyltyramine (FerTyr) – and the phenylamide phytoalexins triticamide A (TriA) and triticamide B (TriB). Lineages by molecular phylogenetic analysis are shown in colour: blue, TauL1; red, TauL2; teal, TauL3.

new MAs biosynthesis. We expect that Fe-deficiency tolerance or Fe biofortification of bread wheat could be generated by introducing *Ae. tauschii* genes into bread wheat to produce additional MAs or increase MA contents.

The root exudates of 42 accessions of *Ae. tauschii* contained mainly DMA (Fig. 1). These results are consistent with previous reports that some accessions of *Ae. tauschii* produce and secrete only DMA (Singh *et al.*, 2000; Tolay *et al.*, 2001). In the exudates
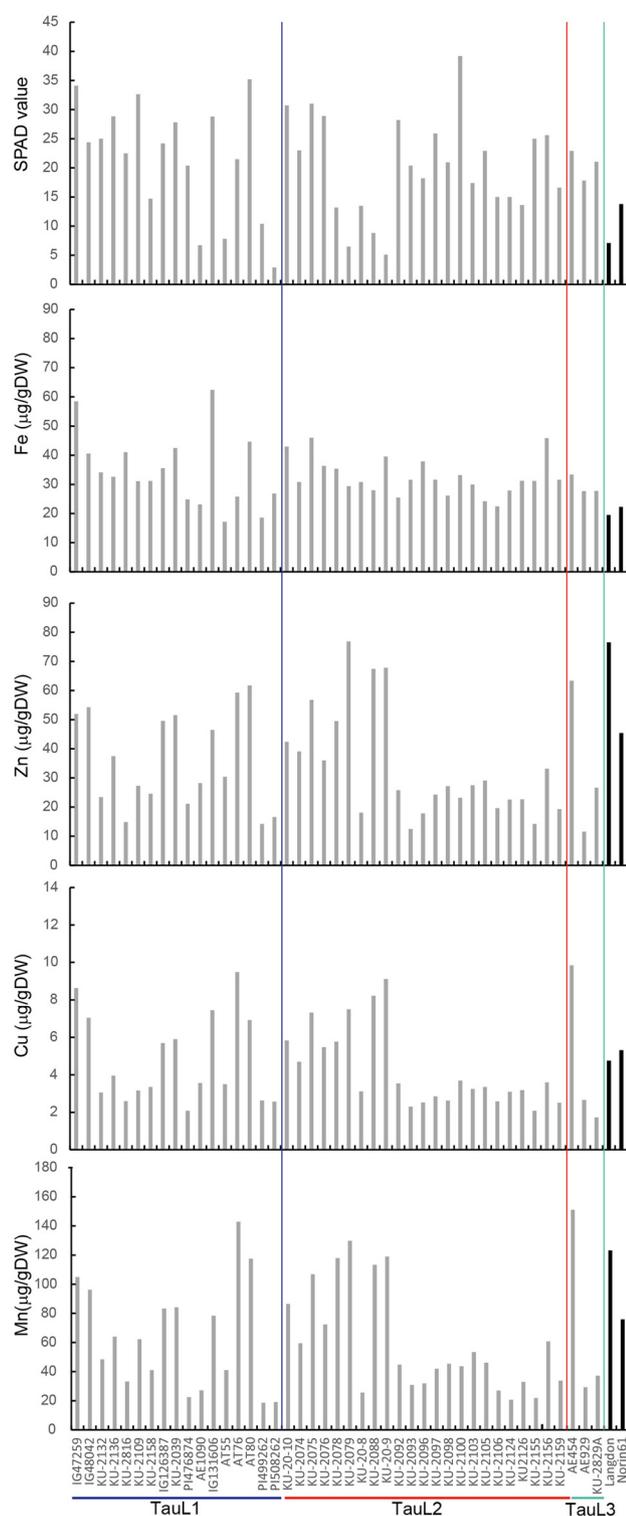
Figure 3. Analysis of SPAD values and concentrations of Fe, Zn, Cu and Mn in shoots of *Ae. tauschii* accessions and wheat 'Norin 61' and 'Langdon'. Lineages by molecular phylogenetic analysis are shown in colour: blue, TauL1; red, TauL2; teal, TauL3.

more kinds of MAs than wheat (Takagi *et al.*, 1984). Since it is speculated that barley was domesticated earlier than wheat and has a different history of breeding, it is hypothesized that few lines of *Ae. tauschii* became ancestors of bread wheat (Ogbonnaya *et al.*, 2013). There are many wild lines of *Ae. tauschii* whose genome is not reflected in bread wheat. Further analysis with more accessions might find accessions that produce and secrete other kinds of MAs.

Although natural variations in *Ae. tauschii* are separated into three lineages (TauL1, 2, 3) by molecular phylogenetic analysis (Sohail *et al.*, 2012; Matsuoka *et al.*, 2013; Mahjoob *et al.*, 2021), the phenotypic traits were not associated with any specific group. Fe solubility differs with soil pH: it is high in acidic soils and low in alkaline soils. TauL3 is found only in Georgia, while TauL2 and TauL1 are found over larger areas (Mahjoob *et al.*, 2021). TauL2 is found mainly along the Caspian Sea in Azerbaijan and Iran. TauL1 is most widespread and is found in Georgia, Turkey, Armenia, Azerbaijan, Syria, Iraq, Iran, Uzbekistan, Tajikistan, Kyrgyzstan, Afghanistan, Pakistan, India and China. Soil pH is alkaline in all of these regions except Georgia (IGBP-DIS, 1998). We used only three accessions of TauL3. Further analysis with more accessions could reveal the connection between Fe homoeostasis in *Ae. tauschii* and soil pH.

Sensitivities to Fe deficiency might differ among *Ae. tauschii* accessions

SPAD values (chlorophyll content) and Fe, Zn, Mn and Cu concentrations in leaves differed among accessions (Fig. 3). Under Fe deficiency, interveinal chlorosis occurs and the SPAD value decreases. It was suggested that chlorosis occurs through the impairment of chlorophyll biosynthesis, since Fe is a cofactor of the several enzyme in this process. The SPAD values and Fe concentrations in leaves were correlated positively with each other but negatively with DMA secretion (Fig. 4a). Fe deficiency transcriptionally induces DMA production and secretion (Kobayashi *et al.*, 2019). These results suggest that sensitivity to Fe deficiency varies among accessions, some of which sense it more strongly than others and enhance MAs production and Fe translocation to the leaves.

MAs may be involved in Fe transport to chloroplasts and may influence the efficiency of photosynthesis (Yoneyama, 2021). Chlorophylls capture solar energy and transfer excited electrons to the photosystem I and II complexes, which contain Fe ions arranged on the chloroplast thylakoids (Raven *et al.*, 1999). Fe concentrations in leaves were positively correlated with Cu, Zn and Mn (Fig. 4a), which are also important in photosynthesis (Yruela, 2013). We speculate that the systems of Fe, Cu, Zn and Mn transport to chloroplasts might overlap.

Phenolics are involved in Fe homoeostasis in *Ae. tauschii*

In Fe-deficient roots, we detected 15 phenylamides and 2 phytoalexins at varying concentrations among accessions (Fig. 2). CouPut, FerPut and CouAgm in the roots were correlated positively with Fe in the leaves (Fig. 4a). Coumarins are involved in Fe uptake from the rhizosphere and Fe translocation in the plant body in *Arabidopsis thaliana* (Rodríguez-Celma *et al.*, 2013; Schmid *et al.*, 2013; Fourcroy *et al.*, 2014; Schmidt *et al.*, 2014). They are biosynthesized via the phenylpropanoid pathway by *ortho*-hydroxylation of cinnamates including Cou and Fer (Shimizu, 2014). In this pathway, *A. thaliana ortho*-hydroxylase

of accessions KU-2109, IG126387, KU-2075, KU-2092, KU-2093, KU-2096, KU-2097 and KU-2156, there were small unknown peaks that differed from known MAs (Fig. 1). These compounds seem to be new MAs, but their amounts were lower than those of DMA, suggesting that their effect on Fe nutrition in the accessions might be small. Barley produces and secretes larger amounts and
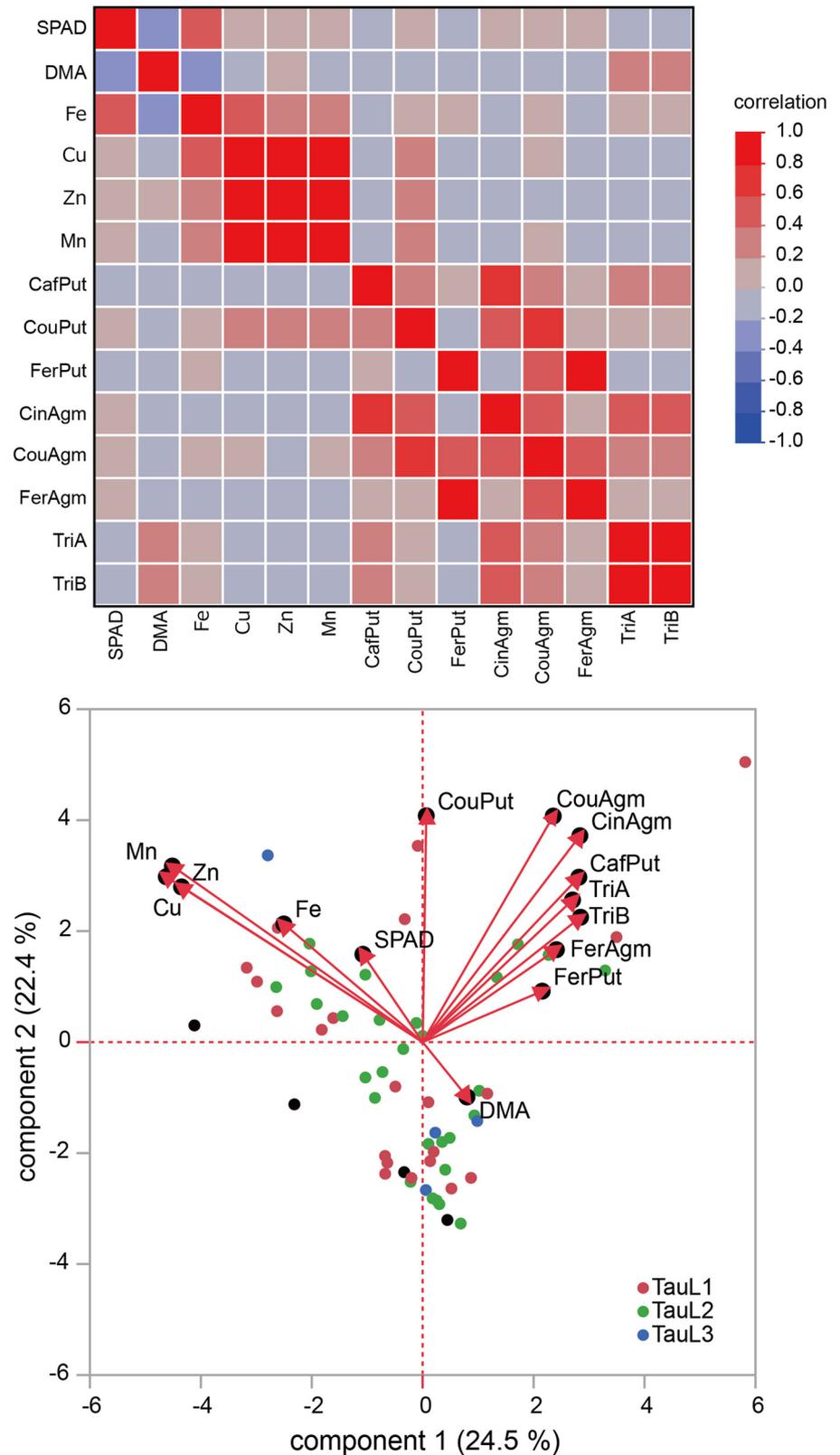
Figure 4. (a) Multivariate analysis and (b) principal component analysis of physiological traits involved in Fe homoeostasis. SPAD, leaf chlorophyll content; DMA, amount of 2′-deoxymugineic acid secreted from roots; Fe, iron in shoots; Cu, copper in shoots; Zn, zinc in shoots; Mn, manganese in shoots; CafPut, caffeoylputrescine in roots; CouPut, *p*-coumaroylputrescine in roots; FerPut, feruloylputrescine in roots; CinAgm, cinnamoylagmatine in roots; CouAgm, *p*-coumaroylagmatine in roots; FerAgm, feruloylagmatine in roots; TriA, triticamide A in roots; TriB, triticamide B in roots. Lineages by molecular phylogenetic analysis are shown in colour: red, TauL1; green, TauL2; blue, TauL3.

(AtF6′H1) is induced at the transcriptional level by Fe deficiency, and its disruption impairs Fe translocation to leaves (Fourcroy *et al.*, 2014). Ferulic acid can also chelate Fe and is effective at rescuing mouse brains from Fe overload (AAlikhani *et al.*, 2022). We

speculate that coumarin and ferulic acid are also involved in Fe homoeostasis in *Ae. tauschii*. Fe concentrations in leaves were positively correlated with both phytoalexins (Fig. 4a). Phytoalexins provide a chemical defence against pathogen infection (Ahuja
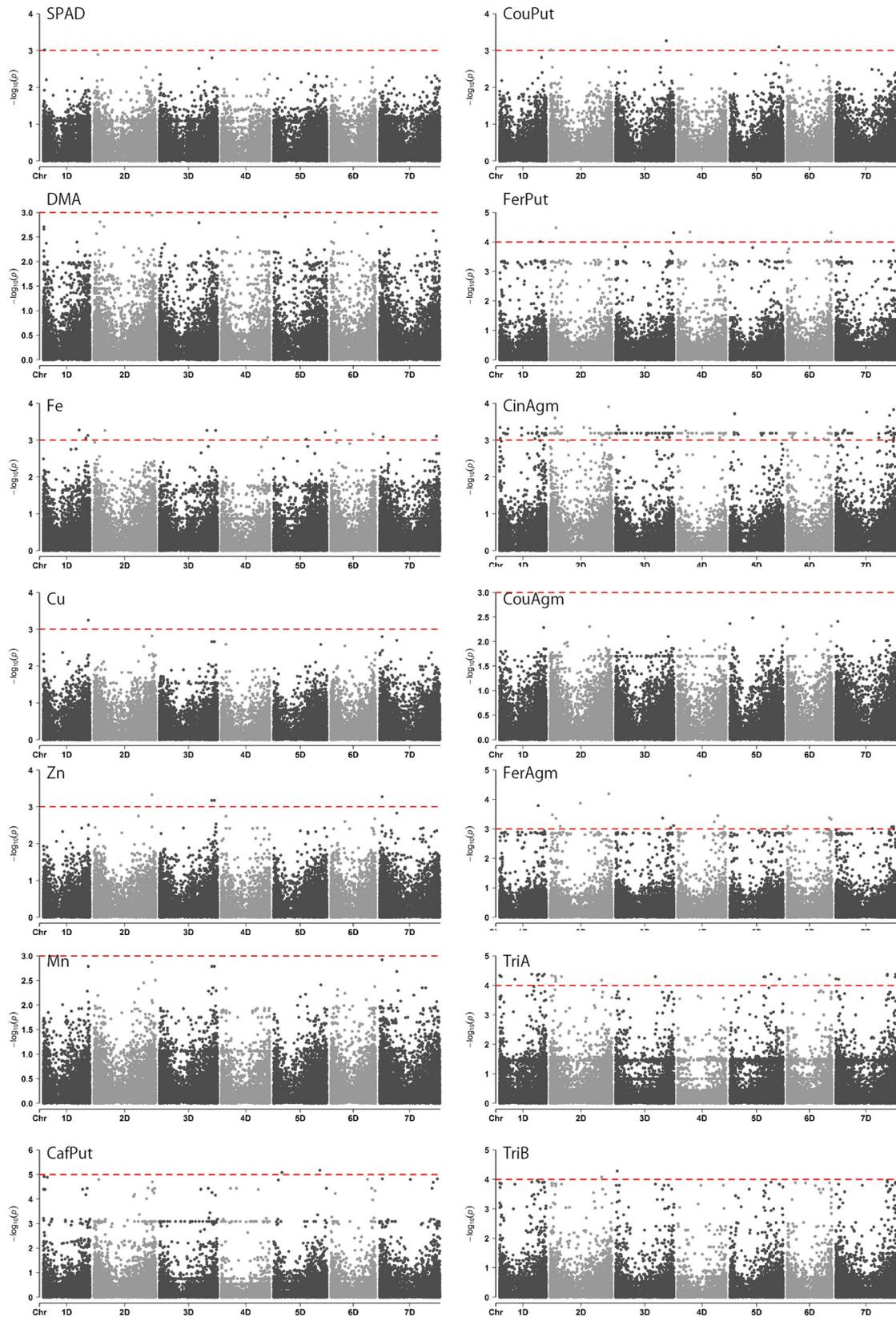
Figure 5. Manhattan plots representing seven chromosomes carrying significant markers detected by Mixed Linear Model using BLUP value in accessions. *x*-axis, genomic coordinates; *y*-axis, negative logarithm of the association *P* value for each single nucleotide polymorphism (SNP). Each dot signifies a SNP.

Table 1. Most significant marker loci-associated traits based on BLUP values in the analysed accessions

Trait	Marker	Chr.	−log10(*P*)		Description
SPAD	4281647	1D	3.02	AET1Gv20067800	Putative F-box/LRR-repeat protein 23
Fe	32785761|F|0-46: C>T-46:C > T	1D	3.28	AET1Gv20678900	Laccase-13 (OsLAC13) like L-ascorbate oxidase (Os05g0458300)
Mn	32782648	7D	2.92	AET7Gv20131100	BTB/POZ and MATH domain-containing protein 3 (BPM3)-like
Zn	32770016	2D	3.33	AET2Gv21156300	ABC-1 domain containing protein (Os04g0640500), Protein kinase superfamily protein (At1g71810)
Cu	32783356	1D	3.25	AET1Gv20974700	Root phototropism protein 2(RPT2)
DMA	32785943|F|0-22: T>A-22:T > A	2D	2.95	AET2Gv21152100	Uncharacterized protein
CafPut	32741419|F|0-63: C>T-63:C > T	5D	5.18	AET5Gv20992700	Conserved hypothetical protein (Os03g0788300)
CinAgm	32720738	2D	3.90	AET2Gv21172900	Benzothiadiazole-inducible diacylglycerol kinase (OsBIDK1) (Os04g0634700)
CouAgm	32750622	5D	2.48	AET5Gv20365900	Glucosamine-fructose-6-phosphate aminotransferase (GFAT) (Os12g0131100)
CouPut	32782989|F|0-26: G>T-26:G > T	3D	3.27	AET3Gv20996200	Tubby, C-terminal domain-containing protein (Os01g0931600)
FerAgm	32781979	4D	4.81	AET4Gv20307400	Not found
FerPut	32772439	2D	4.48	AET7Gv21085000	Not found
TriA	32754846|F|0-13: G>C-13:G > C	1D	4.38	AET1Gv20604600	Putative Rhomboid homolog (OsRhmbd3) (Os01g0283500)
TriB	32763741	3D	4.29	AET3Gv20081800	(1,4)-Beta-xylan endohydrolase, isoenzyme X-II (Os01g0134900)

'Trait': plant phenotype analysed; 'Marker': name of the gene marker; 'Chr': number of the Ae. tauschii chromosome where the marker is located; '−log10(*P*)': the negative logarithm of the association *P* value for each marker values based on BLUPs; 'Overlapping or closest gene': gene located at or closest to the position in Gramene (most similar to the marker in Aet_v4.0: https://www.gramene.org/); 'Description': annotation of the listed gene.

et al., 2012). Triticamides are novel phytoalexins identified in barley and wheat that accumulate in roots in response to microbial infection (Ube *et al.*, 2019a, 2019b). Phytoalexins are regulated by jasmonate (JA) and salicylic acid (SA) (Miyamoto *et al.*, 2014). Triticamides are induced by JA and cytokinins but not by SA (Ube *et al.*, 2019a, 2019b). Endogenous JA rapidly increases in roots in response to Fe deficiency treatment in rice (Kobayashi *et al.*, 2016). Moreover, a basic leucine zipper transcription factor, OsbZIP83, which is involved in Fe homoeostasis, positively regulates genes related to phytoalexin biosynthesis and the SA pathway (Kobayashi *et al.*, 2022). We speculate that triticamides are involved in Fe homoeostasis in *Ae. tauschii* under the control of JA.

Candidate genes in Ae. tauschii *accessions to improve Fe homoeostasis of bread wheat*

The insertion of alien chromosome segments is an attractive method to improve the physiological and agronomic traits of wheat (Ogbonnaya *et al.*, 2013). For example, the improvement of Zn efficiency, the ability to grow and yield better under Zn deficiency, in synthetic wheat by genes from *Ae. tauschii* (DD) suggests the presence of genes for high Zn efficiency in the D genome of *Ae. tauschii* (Cakmak *et al.*, 1999). And the insertion of *Aegilops* chromosomes increased seed Fe and Zn concentrations by 50 to 248%, respectively, relative to the recipient wheat cultivar (Wang *et al.*, 2011). Here, GWAS analysis supported the association of several markers with DMA secretion from roots, leaf SPAD values, shoot metal concentrations and root phenylamides and phytoalexins (Table 1, online Supplementary Table S2).

For SPAD, AET1Gv20067800, annotated as a putative F-box/LRR-repeat protein 23, was closest to the marker. In humans, F-box/LRR-repeat protein 5 (FBXL5) acts as an Fe sensor by degrading iron regulatory protein 2 under Fe-replete conditions through the ubiquitin–proteasome system (Salahudeen *et al.*, 2009; Vashisht *et al.*, 2009). Although plants do not contain this animal Fe-deficiency response system, the protein contains a putative Fe-binding haemerythrin domain as in FBXL5, which acts as an Fe sensor in *Arabidopsis* (Long *et al.*, 2010) and rice (Kobayashi *et al.*, 2013). Since chlorophyll contents in the leaves of Fe-deficient *Ae. tauschii* accessions were diverse and were correlated with Fe deficiency, differences in AET1Gv20067800 might influence susceptibility to Fe deficiency through its role as an Fe sensor in *Ae. tauschii*.

For Fe, AET1Gv20678900, which has homology to Os05g0458300, a laccase-13 (OsLAC13) similar to L-ascorbate oxidase, was closest to the marker. Ascorbate can donate electrons and scavenge free radicals to reduce Fe^{3+} to Fe^{2+} (Smirnoff, 2018). Laccase could be involved in processes that affect Fe homoeostasis such as lignification, ferroxidase activity and oxidative stress, which are speculated to be regulated through microRNAs upregulated under Cu deficiency in *Arabidopsis* and rice (Liu *et al.*, 2017; Carrió-Seguí *et al.*, 2019). AET1Gv20678900 could be involved in Fe accumulation in shoots under Fe deficiency in *Ae. tauschii*.

For Zn, AET2Gv21156300, which has homologies to Os04g0640500, an ABC-1 domain-containing protein, and to AT1G71810, a protein kinase, lay in the marker region. The ABC-1 domain is found in yeast ABC1 proteins required for the function of the mitochondrial bc1 complex (Bousquet *et al.*, 1991), in which ubiquinone functions as an essential cofactor.

Although the function of these proteins is not clear, the ABC-1 domain is found in a eukaryotic-type protein kinase, so a role in the regulation of phosphorylation has been speculated. The ABC-1-like protein AtOSA1 (*A. thaliana* oxidative stress–related ABC1-like protein) is involved in cadmium, oxidative and high-light stresses (Jasinski *et al.*, 2008). AET2Gv21156300 is associated with not only Zn but also Mn and Cu (online Supplementary Table S2), suggesting its involvement in the response to stress caused by metal imbalances.

For Cu, AET1Gv20974700, which has homology to root phototropism protein 2 (RPT2), was closest to the marker. RPT2 is a signal transducer of the phototropic response and photo-induced movements and is involved in adaptation to high-intensity light (Haga *et al.*, 2015). AET1Gv20974700 was also associated with Mn. Since AtOSA1, which is associated with both Cu and Mn, is also involved in responses to high light along with RPT2, high light might be involved in the accumulation of Cu and Mn in *Ae. tauschii* shoots under Fe deficiency.

For Mn, AET7Gv20131100, which has homologies to BTB/ POZ and MATH domain-containing protein 3 (BPM3)-like, was closest to the gene marker. BPM3 acts as a substrate-specific adapter of an E3 ubiquitin–protein ligase complex that mediates the ubiquitination and subsequent proteasomal degradation of target proteins (Weber *et al.*, 2005) and assembles with members of the ethylene response factor/Apetala 2 transcription factor family, which is induced by abiotic stresses (Weber and Hellmann, 2009). AET7Gv20131100 was also associated with Zn and Cu (online Supplementary Table S2), so it might be involved in the induction of gene expression to cope with an imbalance in metal homoeostasis caused by Fe deficiency.

Genes involved in responses to disease and pathogens were included in the list of gene markers that could be related to phenylamides and phytoalexins. For CinAgm, AET2Gv21172900, which has homology to diacylglycerol kinase (OsBIDK1), lay in the marker region. OsBIDK1 has a role in disease resistance responses, and its overexpression enhances disease resistance in transgenic tobacco (Zhang *et al.*, 2008). For CouAgm, AET5Gv20365900, which has homology to Os12g0131100, which is similar to glucosamine-fructose-6-phosphate aminotransferase (GFAT), was closest to the marker. GFAT is the first rate-limiting enzyme of the hexosamine biosynthesis pathway and is suggested to be involved in chitin biosynthesis in insects (Kato *et al.*, 2002), although its function in plants has not been identified.

For CouPut, AET3Gv20996200, which has homology to Os01g0931600 and Tubby (C-terminal domain-containing protein), was closest to the marker. Tubby proteins are found in multicellular plants and animals and are suggested to have a role in transcription (Carroll *et al.*, 2004). GWAS analysis listed Os01g0931600 among markers associated with chilling-tolerant germination in maize (Ma *et al.*, 2022). For TriA, AET1Gv20604600, which has homology to a putative rhomboid homolog (OsRhmbd3), lay in the marker region. Rhomboid-like is a widespread superfamily of intramembrane serine proteases conserved in all kingdoms and is suggested to be involved in signal transduction of several biological functions (Freeman, 2014). For TriB, AET3Gv20081800, which has homology to Os01g0134900, or '(1,4)-beta-xylan endohydrolase, isoenzyme X-II', lay in the gene marker region. '(1,4)-Beta-xylan endohydrolase, isoenzyme X-II' is implied to be involved in cell wall degradation in germinated barley grain and has similarities to pathogenesis-related glucanases in plants that hydrolyse the

polysaccharides that are abundant in fungal cell walls (Hrmova *et al.*, 1997). AET3Gv20081800 was also associated with CinAgm (online Supplementary Table S2). These genes might be involved in the biosynthesis of phenylamides and phytoalexins under Fe deficiency. Further analysis is needed to clarify the functions associated with the markers and their contribution to tolerance to Fe deficiency.

Conclusions

We analysed physiological traits under Fe deficiency in 42 accessions of *Ae. tauschii*. All accessions secreted mainly DMA from their roots under Fe deficiency. Eight of them secreted unknown products, which may be new MAs, from their roots under Fe deficiency, suggesting that there are new genes involved in MAs biosynthesis in *Ae. accessions*. We identified 15 phenylamides and 2 phytoalexins, amounts of which varied among the accessions. Several correlations among traits suggest that these traits are important to cope with Fe deficiency. The GWAS analysis showed that several markers correlated with the examined traits studied, suggesting that *Ae. tauschii* accessions contain novel useful genes to improve Fe nutrition. Further analysis with more accessions is needed to reveal the most effective markers that confer tolerance to Fe deficiency in *Ae. tauschii*, which will lead to the breeding of Fe-deficiency-tolerant and Fe-biofortified bread wheat.

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