

Pleiotropic Effects of *brz*¹

A Mutation in *Pisum sativum* (L.) cv 'Sparkle' Conditioning Decreased Nodulation and Increased Iron Uptake and Leaf Necrosis

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ABSTRACT

Treatment of *Pisum sativum* (L.) cv 'Sparkle' with ethylmethane sulfonic acid produced a stable mutant, E107, which forms few nodules. The mutant allele exhibits other pleiotropic properties including bronze necrotic spots on the leaflets and high accumulation of iron in the shoot. The mutant phenotype is under monogenic recessive control. The gene, designated *brz* (bronze), is nonallelic with two other genes conditioning necrotic spots on leaves of other mutants of *P. sativum*. The *brz* allele was located on chromosome 4 by linkage with wax production controlled by alleles at the *wax* locus.

notably iron which is elevated over fifty-fold above that of the normal parent 'Sparkle' (18). A similar accumulation of iron in pea leaves is reported in the disease purple blight (16), which may occur after prolonged waterlogging of soils. A survey of a *Pisum* collection revealed accessions with necrotic leaves similar to those of E107. These lines were phenotypically compared and crosses among them were analyzed for genetic complementation. We report here that mutant E107 carries a new gene, designated *brz* (bronze), associated with decreased nodulation, increased iron uptake and bronze leaf spots in *P. sativum*.

MATERIALS AND METHODS

Plants

Seeds of the commercial pea (*Pisum sativum*) cultivar 'Sparkle' (S) were a gift from Rogers Bros. Seed Co., Twin Falls, ID.⁴ Seed was treated for 1 h with 1% ethylmethane sulfonic acid (0.08 M). The M₂ mutant selection E107 was found in a search for induced mutants with few or no nodules (8, 9).

Seeds of cv 'Rondo' were obtained from Dr. T. A. Lie, Wageningen, The Netherlands. Seeds of cv 'Dippes Gelbe Viktoria' (DGV), A79-397 (*dgl*), A480-204 and B79-775 (*pub*), and A583-139 (*was*) were obtained from the *Pisum* collection of the late Prof. G. A. Marx, Department of Horticultural Science, New York State Experiment Station, Geneva, NY. Line A79-397 (*dgl*) is an X-ray induced mutant line of 'Dippes Gelbe Viktoria' obtained by Prof. W. G. Gottschalk, University of Bonn, FRG, and is characterized by necrotic leaves and reduced plant size and yield (5). The "*pub*" gene in two differing backgrounds had been obtained originally from L. G. Cruger, Del Monte Research Center, San Leonardo, CA. The mutant was given the provisional symbol *pub* by G. A. Marx (unpublished results) because the leaflets show symptoms similar to those in the disease purple blight (16). *Was*, located on chromosome 4, is one of several

The genetic contribution of the host plant to nodule formation is not as well documented as that of the microsymbionts. To permit application of the techniques of mutational analysis (12), we have induced stable mutants of pea (*Pisum sativum* (L.) cv 'Sparkle') which do not nodulate, form few or ineffective nodules, or have altered strain specificity (8, 9). Most of these mutant plants appear otherwise normal. Some, however, are pleiotropic mutants and display other characteristics not obviously related to nodulation.

One stable mutant line, E107,³ has few nodules at 3 weeks and at that age the lower leaves have small bronze spots. As the plant ages, bronzing appears in upper leaves. The bronze areas enlarge, the leaf tips become brown, and the older leaves die. Leaves were analyzed for mineral content and found to contain larger than normal amounts of several elements, most

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³ Seed of *P. sativum* E107 has been submitted to the Plant Introduction Service of the U.S. Department of Agriculture, Northeast USDA Germplasm Resources, New York State Agricultural Experiment Station, Geneva, NY 14456; to the Wiatrouro Pea Gene Bank, Wiatrouro, 62-100 Wagrowiec, Poland; and to the Nordiska genbanken för jordbruksoch trädgårdsväxter, Box 41, 230 53 Alnarp, Sweden.

⁴ Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Table I. Cosegregation of Leaf Bronzing and Low Nodule Number in F_1 and F_2 Progeny of Crosses between Mutants E107 and 79-397 and normal cultivars 'Sparkle' (S), 'Rondo' (R), and 'Dippes Gelbe Viktoria' (V)

Population	No. of Crosses	No. of Plants	Leaf Phenotype	Nodule Numbers						
				0	1-9	10-29	30-49	50-59	60+	
Sparkle (S)		105	Normal	105				1	3	101
Rondo (R)		13	Normal	13					3	10
DGV (V)		20	Normal	20				2	6	12
E107 (E)		90	Bronze	90	43	36	8	2	1	
A79-397 (D)		42	Bronze	42	1	15	22	4		
S × V, F_1	1	5	Normal	5					2	3
S × E, F_1	11	36	Normal	36					3	33
F_2	7	325	Normal	265				18	17	230
			Bronze	60	37	16	7			
S × D, F_1	3	12	Normal	12				1	2	9
F_2	2	63	Normal	49					1	48
			Bronze	14		4	7	3		
R × E, F_1	1	7	Normal	7						7
F_2	1	79	Normal	59			2	2		55
			Bronze	20	5	5	5	1	3	1
V × E, F_1	2	5	Normal	5					1	4
F_2	2	19	Normal	15						15
			Bronze	4	1	3				
V × D, F_1	4	10	Normal	10				1	4	5
F_2	3	102	Normal	72					12	60
			Bronze	30	1	15	9	3	2	
E × D, F_1	4	21	Spots	21			5	6	5	5
F_2	2	77	Normal	33					5	28
			Spots	19				1	2	16
			Bronze	25	7	6	9	2	1	

loci controlling wax formation and distribution (10). The marker line A583-139 is recessive for *wax* and has shiny (waxless) stipules, whereas 'Sparkle' and E107 are dominant at this locus and have normal dull (waxy) stipules.

Plant Culture

Plants were grown in a light room under high pressure sodium and metal halide lamps with a 16 h/8 h 20 °C/15 °C light/dark regime. For nodule numbers and leaf analysis, peas were planted in coarse vermiculite in 1 inch diameter containers (Ray Leach Cone-tainer Nursery, Canby, OR) and subirrigated. The nutrient medium (9) contained Fe supplied as the citrate complex. In experiments not described here, we observed similar necrotic spots on E107 if Fe in nutrient was provided chelated with EDTA, ethylenediaminedi-O-hydroxyphenyl acetic acid, or diethylenetriaminepentacetic acid.

Seedlings were inoculated 4 d after planting with *Rhizobium leguminosarum* strain 128C53. For growth to maturity, plants were grown in light rooms in commercial planting mix (Redi-earth, W. R. Grace Co., Cambridge, MA).

Genetic Analysis

To test for allelism, reciprocal crosses were made of the two normal cultivars 'Sparkle' and 'Dippes Gelbe Viktoria' and

the four mutant lines. For chromosomal mapping, E107 (*brz*) was crossed with eight *Pisum sativum* (*Brz*) marker lines carrying various marker genes distributed over the *Pisum* genome. Control plants of parental types were grown together with F_1 and F_2 plants. At about 21 d after planting, plants were scored for the presence of bronze leaf spots, and in some experiments for nodule number and leaf Fe concentration. The F_2 progeny from crosses with marker lines segregated for bronze leaf spotting and for morphological or isozyme markers. Nodule number classes were in tens of nodules, and chosen for convenience in counting. The program LINK-AGE-1 (17) was used to compute recombination frequencies.

Fe Analysis

The first or second leaves were oven dried at 60 °C and weighed. Subsamples were ashed overnight at 450 °C, the ash dissolved in HNO₃, and Fe determined by inductively coupled argon-plasma emission spectrometry (18). For graphical presentation and statistical analysis, Fe concentrations were transformed to a log scale.

RESULTS

Selection E107 was observed in an M_2 mutant hunt (9) as a small plant having only few pale nodules. The phenotype

Table II. *F*₂ Segregation Data for Normal (*Brz*/–) and Necrotic (*brz*/*brz*) Leaves in *F*₂ Progeny of Mutant E107 and Cultivars Sparkle, Rondo, Dippes Gelbe Viktoria (V), and Tester Line A583-139

Chi square was calculated using Yates modification for small samples.

Cross E107 ×	No. of <i>F</i> ₂ Plants			Chi Square for 3:1	P
	Total	Normal	Bronze		
cv Sparkle					
Cross #4212	70	58	12	1.90	0.1–0.2
4213	14	12	2	0.12	0.7–0.8
4235	35	26	9	0.01	0.9–0.95
6101	82	70	12	4.16	0.025–0.05
6102	23	17	6	0.01	0.9–0.95
6917	31	26	5	0.87	0.3–0.5
7313	70	56	14	0.52	0.3–0.5
		χ^2 homogeneity (6 <i>df</i>):		2.79	0.7–0.9
cv Rondo	79	59	20	0.004	0.95
A583-139	75	59	16	0.36	0.5–0.7
cv DG Viktoria	19	15	4	0.16	0.9

was stable in *M*₃ and subsequent generations, E107 having fewer nodules than the parent 'Sparkle' grown under the same conditions (Table I). Leaves of E107 are smaller than those of 'Sparkle,' and about 3 weeks after planting (five leaf stage) small bronze areas are observed on leaflets and then the stipules at the third and fourth nodes. As the plant grows, the bronze areas enlarge, the leaflet tips become pale and desiccated, and the leaves die. The symptoms move progressively up the plant as it ages.

Small bronze spots appear on fully filled green pods but the pod matures and dries before spots enlarge. Brown discolored regions are sometimes visible on the roots. Seeds of E107 are viable (>95% germination), contain normal amounts of Fe (average $69 \pm 7 \mu\text{g g}^{-1}$ versus $57 \pm 7 \mu\text{g g}^{-1}$ for 'Sparkle') but are smaller than the parent seeds (average weight 240 ± 63 mg/seed versus 305 ± 81 mg/seed for 'Sparkle').

The multiple characteristics appear to be controlled by the same gene, the low nodulation and leaf bronzing segregating together (Table I). The effect is not restricted to 'Sparkle' (S) genetic background but is also manifested in crosses with cultivars 'Rondo' (R) and 'Dippes Gelbe Viktoria' (V) (Table I). With the exception of one cross, segregation ratios approximate the 3:1 expected for a single recessive gene (Table II). This gene we designate *brz* (bronze) for the color of the necrotic leaf spots.

Segregation data for a cross between E107 and the marker line A583-139 (Table III) confirms that *brz* segregates as a single gene. Significant deviation from random assortment was observed for the joint segregation of *brz* and *was* loci, indicating that it is linked to *was* on chromosome 4. Segregation ratios for *brz* fit the expected 3:1 ratio in crosses with other tester lines (data not shown), but linkage between the leaf necrosis and other morphological or allozyme markers on chromosomes number 1, 2, 3, 5, 6, 7, and 7' was not detected.

The Fe concentration of the first or second leaves is a quantitative measure of *brz* gene expression which was used in further genetic analysis. The Fe concentration in E107 is over 50-fold greater than that of 'Sparkle' (Fig. 1, Table IV). *F*₁ heterozygotes derived from 'Sparkle' × E107 (S × E) are normal in leaf phenotype, but they are intermediate in Fe concentration to that of the parental lines, indicating that the *brz* gene acts as a semidominant gene with respect to Fe concentration. Approximately one fourth of the *F*₂ progeny segregates in a distinct group with the high Fe concentration of E107 (Fig. 2, Table V). The remaining three quarters of the *F*₂ plants have an intermediate Fe concentration, or the Fe concentration characteristic of parental 'Sparkle.' The cosegregation of leaf phenotype, nodule number, and Fe

Table III. Joint Segregation Data for Genes *brz* and *was* in *F*₂ Progeny of Crosses between A583-139 (*Brz was*) and E107 (*brz Was*)

Chi squared values were calculated using Yates modification for small samples.

	<i>N</i>	<i>Brz was</i>	<i>Brz Was</i>	<i>brz was</i>	<i>brz Was</i>
Observed	75	23	36	0	16
Expected	75	14	42	5	14
	<i>Brz</i>	<i>Was</i>	Joint	Recombination fraction ± SE	
* Chi square:	0.36 ^a	1.00 ^a	9.00 ^{b*}	0.01 ± 0.12	

^a Expected 3:1 ratio. ^b expected 9:3:3:1 ratio. * Significant deviation from random assortment, P = 0.01.

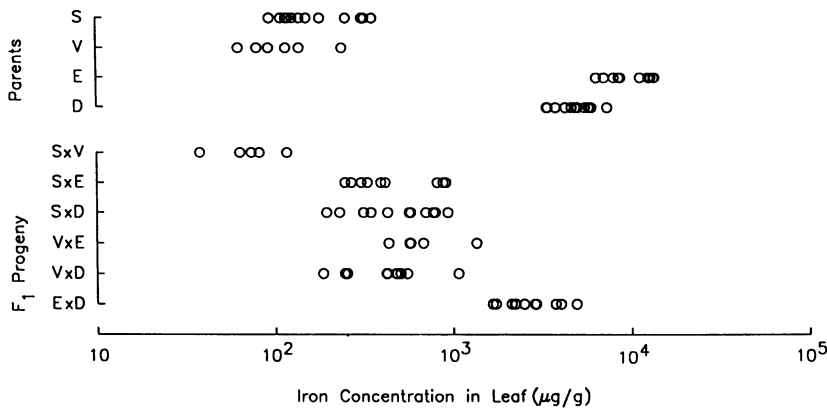


Figure 1. Iron concentration (dry weight basis) of leaves of 'Sparkle' (S), 'Dippes Gelbe Viktoria' (V), the mutants E107 (E) and *dgl* (D), and F₁ progeny from their reciprocal crosses.

concentration (Table V) is evidence for a single gene with pleiotropic effects.

Mutant line A79-397 (*dgl*) also is poorly nodulated and has a high Fe concentration relative to its parent 'Dippes Gelbe Viktoria.' In A79-397 crosses both with 'Sparkle' (S × D) and with DGV (V × D), F₁ heterozygotes have an intermediate Fe concentration noticeably higher than that of the normal controls, but lower than that of the mutants (Fig. 1, Table IV). The F₁ heterozygotes do not, however, have leaf spots (Table I). Again, the bronze spot, low nodulation, and high Fe concentration cosegregate in the F₂ as a single gene (Tables I and V, Fig. 2). The Spearman rank correlation for leaf phenotype and nodulation is $r = 0.79$, while that for leaf phenotype and iron content is $r = -0.85$. The Pearson Product moment correlation for nodulation and iron content is $r = 0.88$.

The cross between the two iron accumulating lines, E107 and *dgl* (E × D), yields a F₁ population with a unique phenotype (Table I, Fig. 1). Small yellow spots appear on the leaves, but they do not enlarge to the bronze necrotic symp-

toms seen in the mutant lines themselves. The Fe concentration is lower than in either mutant alone, but is significantly higher than that of either the *Dgl*, *dgl* or *Brz*, *brz* heterozygotes alone (Table IV). The two genes partially complement one another, indicating that they are not alleles at the same locus, but interact physiologically.

The F₂ population derived from reciprocal crosses between mutants E107 and A79-397 (E × D) appear to segregate for two genes based on leaf Fe concentration, but distinct classes could not be defined. If *brz* and *dgl* were allelic, the F₂ progeny would all have bronze leaf spots. Segregants with normal leaves and normal Fe concentration are recovered in the F₂, confirming that *brz* and *dgl* are not allelic (Table V). 19 of 77 F₂ segregants displayed an intermediate phenotype of small yellow leaf spots, as had the E × D F₁ plants (Table I). Furthermore, these F₂ progeny had a unique intermediate Fe concentration not found in normal (S or V) × mutant (E or D) crosses (Table V, Fig. 2). The *brz* and *dgl* genes both act semidominantly resulting in a unique phenotype for double heterozygotes.

The two lines carrying the *pub* gene had purple-black leaf flecks which appeared later than the bronze spots on the leaves of *brz* and *dgl* lines. They had normal Fe concentrations in leaves; averaging 179 and 127 $\mu\text{g Fe g}^{-1}$ dry weight compared with 183 $\mu\text{g g}^{-1}$ dry weight in the lower leaves of 'Sparkle.' Analysis of other ions in leaves (data not shown) did not indicate any abnormality in the mineral nutrition of the *pub* leaves. Crosses of the *pub* lines with E107 and A79-397 did not indicate allelism or interaction for Fe content or leaf necrosis (data not shown).

Table IV. Iron Concentration in First or Second Leaf of Normal Pea Cultivars "Sparkle" (S) and "Dippes Gelbe Viktoria" (V), Mutant Lines E107 (E) and A79-397 (D), and F₁ progeny of Their Reciprocal Crosses

The data were transformed to a logarithmic scale (Figs. 1 and 2) and analyzed by Duncan's multiple range test

Population (Genotype)	N	Fe	Log Mean ^a
		$\mu\text{g g}^{-1}$ dry wt	
Parents			
(S)	13	183 ± 92 ^b	5.11 F
(V)	6	122 ± 64	4.70 G
(E) (<i>brz/brz</i>)	13	10160 ± 2690	9.19 A
(D) (<i>dgl/dgl</i>)	13	5020 ± 1150	8.50 B
F₁ Progeny			
S × V	5	75 ± 29	4.25 H
S × E (<i>Brz, brz</i>)	10	547 ± 290	6.17 D, E
S × D (<i>Dgl, dgl</i>)	12	516 ± 248	6.13 D, E
V × E (<i>Brz, brz</i>)	5	724 ± 365	6.50 D
V × D (<i>Dgl, dgl</i>)	10	462 ± 248	6.02 E
E × D (<i>Brz, brz; Dgl, dgl</i>)	21	3030 ± 1100	7.96 C

^a Means with the same letter are not significantly different. ^b ± = standard deviation.

DISCUSSION

We have genetically characterized *brz*, a mutant gene of pea which, in the homozygous recessive form, pleiotropically conditions decreased nodulation and necrotic bronze areas on leaflets (Table I) and a high concentration of Fe in the shoot (Figs. 1 and 2; Tables IV and V) (18).

Three lines with leaf necrosis were found in searching a list of pea genes (2) and in the collection of *Pisum* mutants maintained at the New York State Experiment Station, Geneva, NY. Two lines carrying *pub* had necrotic leaf spots, but the mineral content of their leaves was normal.

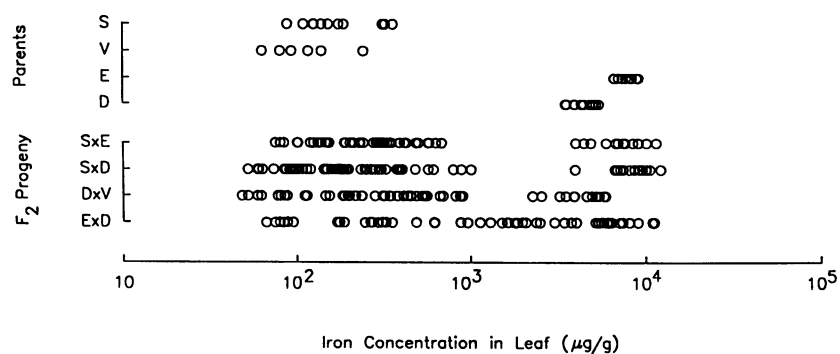


Figure 2. Iron concentration (dry weight basis) of leaves of 'Sparkle' (S), 'Dippes Gelbe Viktoria' (V), the mutants E107 (E) and *dgl* (D), and F₂ progeny from their reciprocal crosses.

Another mutant line with bronze leaf necrosis, A79-397 (*dgl*), contained an abnormally high leaf Fe concentration (Figs. 1 and 2; Table IV) and had reduced nodule numbers. Although *dgl* was discovered two decades ago (5), its physiology has not been studied in detail.

Complementation tests indicate that *brz* and *dgl* are not allelic (Tables I, IV, and V; Figs. 1 and 2). If the genes were

allelic, all the F₁ and F₂ progeny would have had low nodulation, bronze spots, and been high in Fe. The Fe concentration in F₁ heterozygotes was intermediate (Table IV), suggesting that these genes are semidominant. It was not possible to distinguish heterozygotes within the F₂ population due to the variability in Fe concentration.

The leaf lesions appear similar to published descriptions of

Table V. Cosegregation of Leaf Phenotype, Nodule Number, and Fe Concentration in Leaf of F₂ Progeny of Crosses between Mutants E107 (*brz*) and A79-397 (*dgl*) and Normal Cultivars 'Sparkle' (S) and 'Dippes Gelbe Viktoria' (V)

Each cell contains the number of plants and their Fe concentration in $\mu\text{g g}^{-1}$ dry weight (mean \pm standard deviation).

Population Parents	Leaf	No. of Plants	Nodule Number		
			0-29	30-59	60+
Sparkle (S)	Normal	11	0	2	9
				106 \pm 25	207 \pm 94
Dippes Gelbe Viktoria (V)	Normal	6			6
					121 \pm 63
A79-397 (<i>dgl</i>) (D)	Spots	11	10	1	0
			4280 \pm 663	4828	
E107 (E) Progeny S \times E F ₂	Bronze	11	11	0	0
			7514 \pm 817		
	Normal	46	0	7	39
				480 \pm 152	245 \pm 118
S \times D F ₂	Normal	48	0	1	47
				180	240 \pm 192
	Spots	2	1	0	1
			3846		869
Bronze	13	10	3	0	
		8766 \pm 1623	8027 \pm 1416		
V \times D F ₂	Normal	44	0	0	44
					316 \pm 223
	Bronze	16	11	3	2
			4537 \pm 1060	4015 \pm 763	3651 \pm 2053
E \times D F ₂	Normal	22	0	2	20
				933 \pm 464	279 \pm 247
	Spots	18	0	3	15
				3190 \pm 2234	1657 \pm 770
Bronze	20	17	3	0	
		6872 \pm 2116	7371 \pm 3565		

the "bronzing" in rice and the "freckle leaf" of sugar cane associated with Fe toxicity (4). Such iron toxicity usually originates with soil flooding, and excessive uptake of other elements including Al, Mn, and Zn may also occur. The metabolic defect responsible for the leaf necrosis is not known in crop systems or in the mutant pea lines examined here.

The extraordinary uptake of Fe reported here is novel. This is the first report linking individual plant genes, *brz* and *dgl*, to enhanced iron uptake. In reviewing phytotoxicity of metals, Foy *et al* (4) note that there are intergeneric and interspecific differences in tolerance to metal toxicity. Similarly, soybean cultivars may differ in how well they take up Fe from soil. 'Iron efficient' cultivars may accumulate up to twice as much Fe as 'iron-inefficient' cultivars growing on calcareous soils. Resistance to iron chlorosis in soybeans is a quantitative trait, and apparently involves many genes (1, 3, 6). In no previous study has genetic analysis implicated a single plant gene in increased Fe uptake.

A mutant line of tomato (*Lycopersicon esculentum*) T3238 (*fer fer*) is inefficient in absorbing Fe compared with normal (*Fer*) genotypes. The single gene mutation results in lower than normal concentrations of caffeic acid and *p*-coumarate hydroxylase in roots of the tomato mutant (13).

A similar genetic defect in iron metabolism in humans is the disease idiopathic hemochromatosis (7). Originally called bronze diabetes, this disease involves enhanced iron accumulation, ultimately leading to toxic levels in parenchymal cells of the liver, heart and pancreas (14). Genetic analysis of families of patients show that it is conditioned by a homozygous recessive gene.

Most E107 plants have no or very few nodules. A few have an intermediate number (Table I), just as some occasional 'Sparkle' plants have fewer nodules than normal. Such variability in nodulation, not unique to this study, frustrates researchers using nodule number in genetic analysis.

It is still speculative whether decreased nodulation is directly related to the high Fe concentration. Iron certainly plays an important role in nodule function. The Fe requirements of the nodule for the abundant proteins leghemoglobin, nitrogenase, hydrogenases and ferredoxins suggest that, among plant organs, the nodule has an unusual if not unique demand for iron.

The uptake of Fe by roots involves the secretion of a reductant to convert Fe³⁺ to Fe²⁺, H⁺ ions to solubilize Fe³⁺ ions, and phytosiderophores to chelate the Fe³⁺ (1, 11, 15). If any or all of these substances are secreted in excess, concomitant to the high Fe uptake, then nodulation might be suppressed because the rhizosphere is inhospitable to the rhizobia.

Possibly the excessive uptake of Fe is not the direct cause of the decreased nodulation in E107. In any case, comparative

studies of E107 to its near-isogenic parent 'Sparkle' now provide us with a novel tool for studying not only nodulation but also the plants' uptake and metabolism of Fe.

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