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# Iron Nutrition, Oxidative Stress, and Pathogen Defense

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Additional information is available at the end of the chapter

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## Abstract

Adaptation is a challenge that plants have to undergo in order to survive in difficult environments. Nutrient deficiency, stress, and microorganism attack are abiotic and biotic factors that frequently impair plant wellness, which is reflected by low crop yield and quality. Poor crops in turn affect human nutrition. To solve these problems, it is necessary to understand the molecular and physiological mechanisms of nutrient uptake and adaptation to stress. With this knowledge, we may have the possibility to generate new plants, which offer better yield due to their better health. This chapter summarizes and compares iron uptake and assimilation as well as pathogen responses in plants and humans. We also discuss novel approaches for improving crops in the context of human food quality.

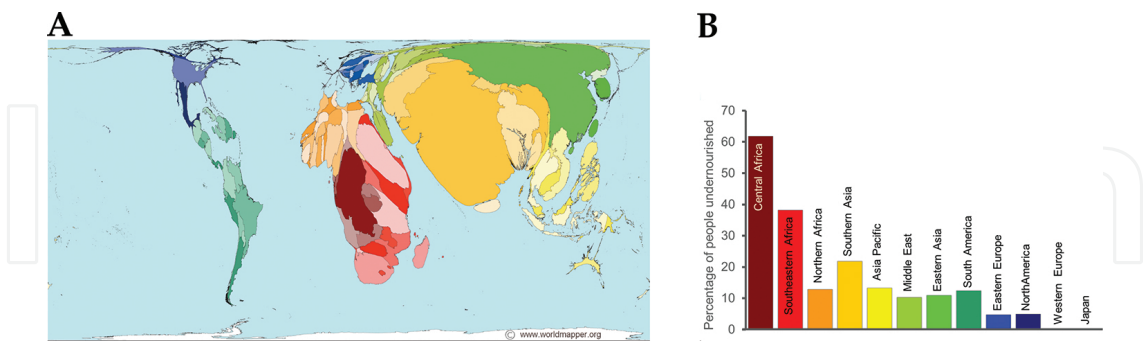
**Keywords:** Iron uptake/absorption, Breeding, ROS, Pathogen

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## 1. Introduction

Organisms have specific ways to assimilate necessary nutrients. Animals, including humans, have to ingest food and process it mechanically and chemically during the digestion. The principal nutrients needed by animals, such as carbohydrates, lipids, proteins, vitamins, and minerals, are found in different sources [1]. A balanced alimentation is hence very important. Lack of essential vitamins or minerals in the diet affects immunity and healthy development. This condition of unproportional alimentation is called undernourishment. Nutrition problems have always been an issue in third world or developing countries. Nowadays, about 104 million children worldwide are underweight (2010). The WHO has a project to reduce by 40% the number of children that are stunted due to the undernourishment until 2025. Currently, in Central Africa over 60% of the population is undernourished, followed by Southeastern

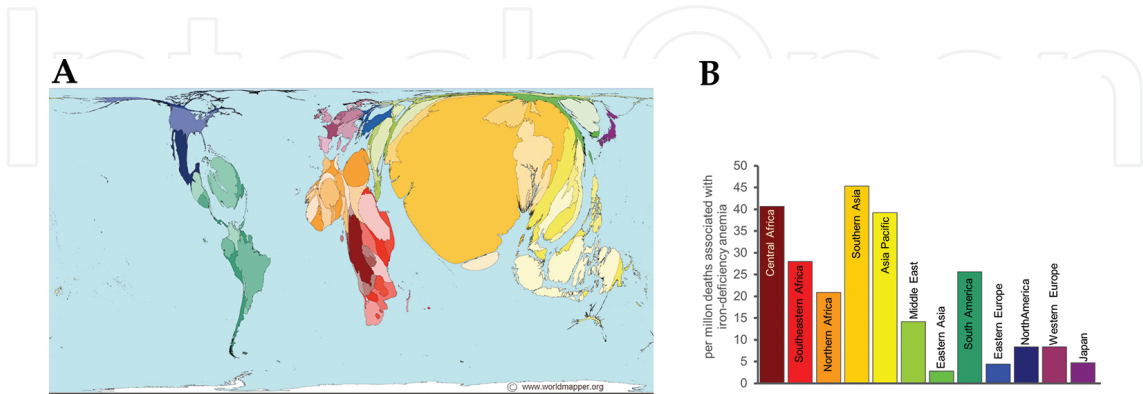
Africa (~40%) and Southern Asia (~20%), which are the regions most affected by undernourishment [2, 3] (**Figure 1**).



**Figure 1.** Worldwide map of undernourishment (2000). (A) The map illustrates the resized world corresponding to the number of under-nourished people living in the different regions. (B) The chart shows the percentage of undernourished people living in the different regions. Africa and southern Asia are the most affected regions with almost 50% of all undernourished people worldwide (adapted from [3], Map 178).

In regions with high undernourishment, many people do not have access to a varied diet and their main alimentation consists in only one specific sort of crop. In theory, crops can be fortified by increasing the level of nutrients or uptake-promoting substances in the soil. For example, raising the supply of the essential micronutrient elements Zn, Ni, I, and Se increases their concentration in the grains of several plant products [4]. Unfortunately for other micronutrients, such as Fe, the sole supplementation of the soil with Fe salts is not sufficient to step up the iron quantity in the crops. Foliar fertilization is the best way to increase Fe content in crops, but the cost and effort are not economically interesting [5–7].

Fe deficiency in the form of anemia affects more than 2 billion people on the planet, being the most common nutritional problem in the world (WHO 2005), and regions with Fe deficiency anemia coincide with those of undernourishment, particularly Asia, Africa, and Latin America (compare **Figures 1** and **2**).



**Figure 2.** Iron deficiency anemia deaths. (A) The size of the regions corresponds to the number per million deaths associated with Fe deficiency anemia (2002). (B) The chart shows the number of people per million inhabitants living in the different regions. Africa, Asia, and South America are the most affected (adapted from [3], Map 414). (adapted from [3], Map 414).

Due to the poor conditions and the lack of access to diverse nutritious food, it is difficult to counteract this problem by just supplementing the food with iron. Genetic engineering is a suitable approach to fortify plants with organic nutrients. In the case of Fe, it is important to not only find a way to increase the efficiency of the uptake into the plant but also the transport inside the crop, and more importantly to improve the bio-availability of Fe for assimilation in humans [8, 9]. An attractive source and plant crop have to be chosen. For example, cassava or manioc (*Manihot esculenta*) is extensively used by humans for food, livestock, and extraction of starch. Manioc can be cultivated for over 30 years in the same field without fertilizer even in poor soil conditions [10, 11]. Besides, roots can be conveniently stored and also remain in the soil for a long time. Although cassava is one of the basic foods for around 800 million people in the world, it is not a good source for iron and other nutrients [12]. Using genetic engineering, [13] researchers were able to introduce a green algae gene (*FEA1*) in cassava and thus increase the storage of iron in the roots from 10 to 36 ppm. This amount of iron would cover the daily requirements for an adult in a meal of 500 g.

In humans, the iron absorption is strictly regulated. Iron overload is not caused by the consumption of high-iron diets but more by an inadequate ingestion of iron supplements or genetic defects in the regulation of iron homeostasis and iron overload diseases [14]. Difficulties in iron homeostasis produce reactive radicals, better known as reactive oxygen species. Such radicals are capable of damaging almost every molecule in living cells such as DNA, lipids, membrane proteins causing various diseases in humans including vascular diseases and cancer [15].

Iron is an essential element for animals, bacteria, fungi, and plants and similar disease problems as in humans may be found. A competitive situation may arise between organisms when they live in close relationship. This is interesting in host–pathogen interaction systems, where a competition for nutrients between host and pathogen is a determinant for an effective immune system and can affect susceptibility and resistance to a pathogen [16].

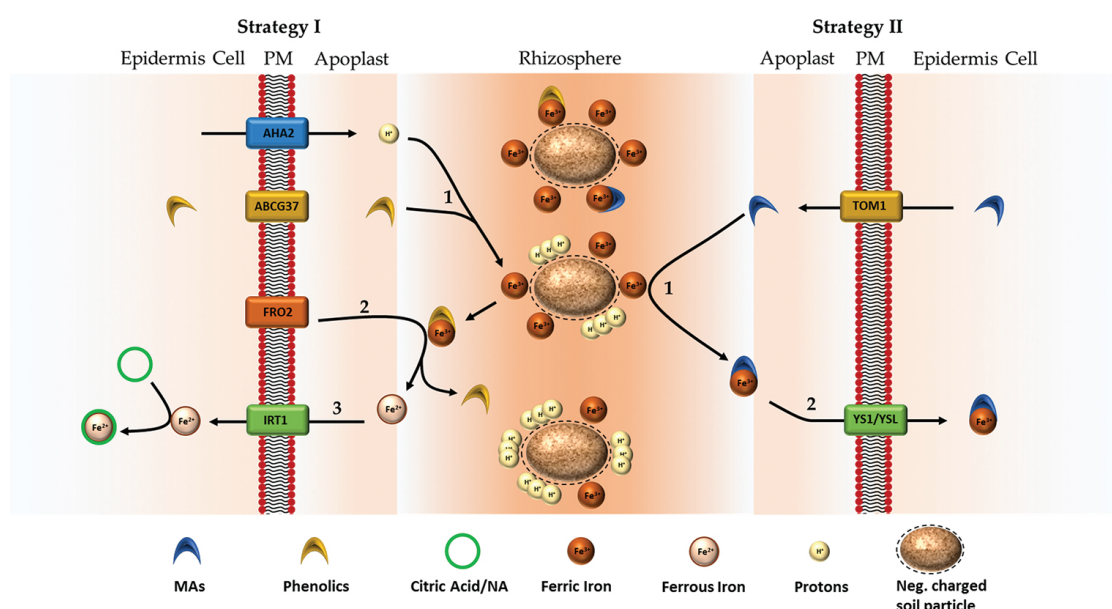
## 2. Iron uptake in plants

Depending on the composition of soil particles, the soil can have different characteristics, some of which define it as fertile. The soil should provide a wide microorganism population, and for most crop plants, a soil pH around 5.5 and 7 is ideal due to the availability of nutrients. The texture of the soil is crucial for the aeration, irrigation, and adequate root proliferation. Its texture is characterized by the amount of sand, silt, and clay particles. High amount of clay particles is necessary to retain essential nutrients and for soil humidity [17]. Around 5.6% of the Earth's crust consists of iron (Fe), belonging to the five most abundant elements. However, the bioavailability of this metal is restricted and plants developed strategies for its mobilization [18]. Iron is a transition metal, and its valence electrons are present in more than one shell so that atoms can be present in several oxidation states [19]. In the nature, iron is present in two biologically important forms, the ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) form. In acidic environment, iron acts as a reducing factor, whereas in basic medium, it acts as an oxidizing agent [20]. In soil,  $\text{Fe}^{3+}$  predominates and is attached to silicate structures and hydroxides. In order to take

up the Fe ions, plants have strategies to dispatch iron from soil particles, chelate or reduce it and transport it into the plant root cells.

## 2.1. Strategies of iron uptake in plants

Regarding iron uptake, land plants can be separated into two main groups: Strategy I and Strategy II plants. All plants, except grasses, carry out Strategy I iron uptake. Among them are, for example, tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), and the model organism *Arabidopsis thaliana*. Strategy II plants are all sweet grasses including rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat (*Triticum* spp.). Studies demonstrated that iron uptake was more efficient in barley (*Hordeum vulgare*) than in cucumber (*Cucumis sativus*) especially at higher pH, which would give Strategy II plants an advantage over Strategy I plants [21].



**Figure 3.** Strategy I and Strategy II iron acquisition in plants. Strategy I plants, exemplified by *Arabidopsis thaliana* (left side), take up iron in three steps: first, in order to liberate Fe<sup>3+</sup> ions, the proton pump AHA2 acidifies the rhizosphere. The secretion of phenolic compounds through the ABCG37 transporter increases the solubilization of iron. Second, the iron reductase FRO2 reduces Fe<sup>3+</sup> to Fe<sup>2+</sup> that finally is transported into the epidermis cell by the iron transporter IRT1. Inside of the plant citric acid or nicotianamine chelate Fe<sup>3+</sup>/Fe<sup>2+</sup> for further transport within the plant via xylem or phloem. The iron uptake in strategy II plants, exemplified by *Zea mays* and rice (right side) consists of two steps: firstly, TOM1 exports phyto siderophores into the rhizosphere to solubilize Fe<sup>3+</sup> ions. The Fe<sup>3+</sup>/PS complex is transport by the YS1 protein in maize and YSL in other grasses.

In both strategies (Figure 3), the proteins required for iron uptake are located in the root epidermis cells [21]. Strategy I plants acidify the rhizosphere by pumping protons, carried out by a proton-ATPase [22]. FERRIC REDUCTASE OXIDASE (FRO2 in *A. thaliana*; LeFRO1 in tomato) is responsible for the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> which is a crucial step for the iron uptake in Strategy I plants [23–25]. In both strategies, roots enhance iron mobilization by secreting iron-chelating compounds [26]. Among these, many phenolic compounds and flavins are found in Strategy I plants. [27]. The investigation of the effect of phenolic compounds in red



clover (*Trifolium pratense*) showed that the excretion of these molecules is important for the reutilization of apoplastic iron by decreasing the mobilization of iron from roots to shoots [28]. Studies in *A. thaliana*, *Brassica napus*, and *Medicago truncatula* demonstrated that these compounds are related to coumarins such as scopoletin and other derivatives as well as flavins. They are produced under iron deficiency conditions, among others, via the action of the feruloyl-CoA 69-hydroxylase1 (F6'H1). Subsequently, the ABC transporter called ABCG37 transports these compounds to the rhizosphere [29–32]. The response of the roots of grasses (Strategy II plants) to iron deficiency is to secrete phytosiderophores (PS) through the phytosiderophore efflux transporter TOM1 [33]. PS are high-affinity iron chelating compounds able to chelate and solubilize ferric iron ( $\text{Fe}^{3+}$ ). The most well-known PS are members of the mugineic acid family (MA) and arvenic acid (AA) [34]. Nicotianamine synthase (NAS) is an important enzyme that catalyzes the fusion of three S-adenosyl methionine molecules (SAM) to form the MA precursor nicotianamine (NA), a non-proteinogenic amino acid [35].

In maize, the first highly specific proton-coupled PS transporter identified was the yellow stripe 1 (YS1). It transports the  $\text{Fe}^{3+}$ /PS as well as  $\text{Fe}^{3+}$ /NA complex into the cells [36–38]. Further investigation revealed closely related transporters, yellow stripe 1-like (YSL), in barley and rice [39–41]. The last step of iron uptake in Strategy I plants is the transport of reduced/chelated Fe handled by the IRON-REGULATED TRANSPORTER 1 (IRT1) [42–44].

In contrast to the other Strategy II plants, rice represents a special case because this plant has the ability to take up both  $\text{Fe}^{3+}$ /PS and  $\text{Fe}^{2+}$  from the soil. Rice produces lower amounts of PS (2'-deoxymugineic acid DMA) than other grasses, but has two genes encoding for proteins similar to the *Arabidopsis* IRT1, OsIRT1, and OsIRT2. OsIRT proteins were found to be located in the root plasma membrane, and they are able to transport  $\text{Fe}^{2+}$ . However, rice plants are usually not forced to reduce iron before transport because they grow in submerged conditions where  $\text{Fe}^{2+}$  is more abundant than  $\text{Fe}^{3+}$  [45].

Once iron enters the symplast of the epidermal root cells, it diffuses across the plasmodesmata to reach the vascular tissues. The IRON-REGULATED PROTEIN 1 (also known as ferroportin FPN1) IREG1/FPN1 loads Fe into the xylem [46]. The root-specific protein FERRIC REDUCTASE DEFECTIVE 3 (FRD3) mediates the efflux of citrate into the xylem. There, citrate chelates Fe and this complex is transported with the transpiration stream to the upper parts of the plants [47–49]. In order to reach developing organs where the xylem is not yet formed, for example, meristem of young leaves or seeds [50], the Fe is loaded into the phloem and chelated with NA [51–53]. Potential transporters of iron between leaves and sinks are the OLIGOPEPTIDE TRANSPORTER 3 (OPT3) [54] and YSL proteins [55]. In *Arabidopsis*, NA-chelated Fe may be transported from the phloem to flowers and seeds via the AtYSL1 and AtYSL3 transporter [56, 57], and in rice, this is performed by OsYSL2 [58].

Immediately after reaching the tissue of destination, Fe has to be stored in cell compartments where utilization and storage need to be coordinated. The Fe-transporter FPN2 and VACUOLAR IRON TRANSPORTER 1 (VIT1) [59] are responsible for import of iron into the vacuole, while NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 and 4 (NRAMP3 AND NRAMP4) mediate its export [60, 61]. Probably, in the vacuoles, iron is chelated with phytates. Photosynthesis, the electron transport chain and synthesis of chloro-

phyll require an enormous amount of Fe. Therefore, the majority of iron is supplied to the chloroplasts [62]. The transport of Fe into the chloroplast requires first its reduction mediated by FRO7, followed by its transport performed by the transporter PERMEASE IN CHLOROPLAST 1 (PIC1) [63, 64]. In the chloroplast, Fe is sequestered in ferritin (FER), which is macroprotein complexes able to store up to 4500 iron atoms and present in animals, plants, fungi, and bacteria [65].

## 2.2. Regulation of the iron uptake in plants

Many transcription factors are responsible for the proper iron homeostasis. The main regulator of the iron uptake is in *A. thaliana* the basic helix-loop-helix (bHLH) protein FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (AtFIT) [66–68] and in tomato the LeFER [69, 70]. FIT and FER interact with the bHLH proteins of the subgroup Ib and with SlbHLH069, respectively, to activate the transcription of the Fe reductase and the Fe transporter genes in roots [71–73]. The *Arabidopsis* bHLH Ib subgroup transcription factors comprise bHLH038, bHLH039, bHLH100, and bHLH101 [74], which share partial redundant functions in iron homeostasis [75]. There is a large number of genes regulated by FIT and iron deficiency [66, 76, 77]. FIT-dependent genes, partly also under regulation by other iron-regulated transcription factors, are *IRT1*, *FRO2* [67], *KELCH REPEAT PROTEIN*, *MTPA2*, *CYP82C4*, among others [78]. In contrast, the four bHLH subgroup Ib genes are not regulated by FIT. Their high transcript levels in the *fit-3* mutant compared to the wild type are rather due to the iron deficiency [75]. These genes are co-regulated with other known iron homeostasis FIT-independent genes such as *PYE*, *BTS*, *FRO3*, *NRAMP4*, and *NAS4* [66, 79–81].

POPEYE (PYE) and BRUTUS (BTS) are tightly related to iron homeostasis. Both genes are upregulated at –Fe conditions; however, they have opposite functions. PYE acts as a transcription factor positively regulating iron status of the plant, while BTS has repressing effects on the iron homeostasis [82]. BTS belong to the RING E3 ligases proteins which have a hemerythrin group and are able to bind Fe and Zn [83]. Both BTS and PYE interact with IL3, bHLH104, and bHLH115. This interaction might occur, according to the requirements to fine-tune iron homeostasis [82, 84].

Other important regulators of the iron uptake are the redundant MYB10 and MYB72 transcription factors, which are upregulated under low iron conditions. *MYB72* counts as a direct target of FIT and regulates *NAS4* and *NAS2* [81, 85, 86]. Furthermore, MYB72 regulates also the transcription of *BGLU42* that is involved in the production of phenolic compounds, which are excreted by the root to mobilize iron from insoluble sources [30, 81, 87].

## 3. Iron absorption in humans

In healthy humans, iron represents around 40 mg/kg body weight [88]. Most iron contained in the human body (70%) is circulating with the erythrocytes in form of hemoglobin, around

10% in the muscles as myoglobin, cytochrome, and iron-containing enzymes and the residual 20% as ferritin [89, 90]. The daily-recommended iron dosage for healthy adults is 8 mg for men, 18 mg for women, and 27 mg for pregnant women [91]. Iron absorption efficiency varies depending on the iron type (heme iron or nonheme iron), iron content of the food, iron status of the body, and consumption of iron-absorption inhibitors or enhancers [92]. Meat, poultry, and fish contribute to heme iron while all vegetables, cereals, and legumes with the inorganic oxidized ferric form ( $\text{Fe}^{3+}$ ) (nonheme iron). The bioavailability of heme iron is around fivefold better than nonheme iron, even though the iron content of some plant aliments is much higher than animal food sources [93, 94]. Iron-rich plant aliments are often derived from leafy green vegetables because chloroplasts contain high amounts of metalloproteins that function in the electron transport chain. Seeds or whole grains can also be a good source of iron, which is stored in the form of iron phytate or ferritin in the seed coat or embryo [95].

Many substances consumed simultaneously such as phytic acid and polyphenols impair the bioavailability of nonheme iron [96–98]. Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) constitutes the principle phosphorus compound in seeds. Under physiological conditions, these strongly negative compounds form salts with cations such as Ca, Zn, Fe, or Cu to form phytates. In seeds, these salts are found principally in the aleurone layer providing sufficient nutrient sources for the germination [96, 98, 99].

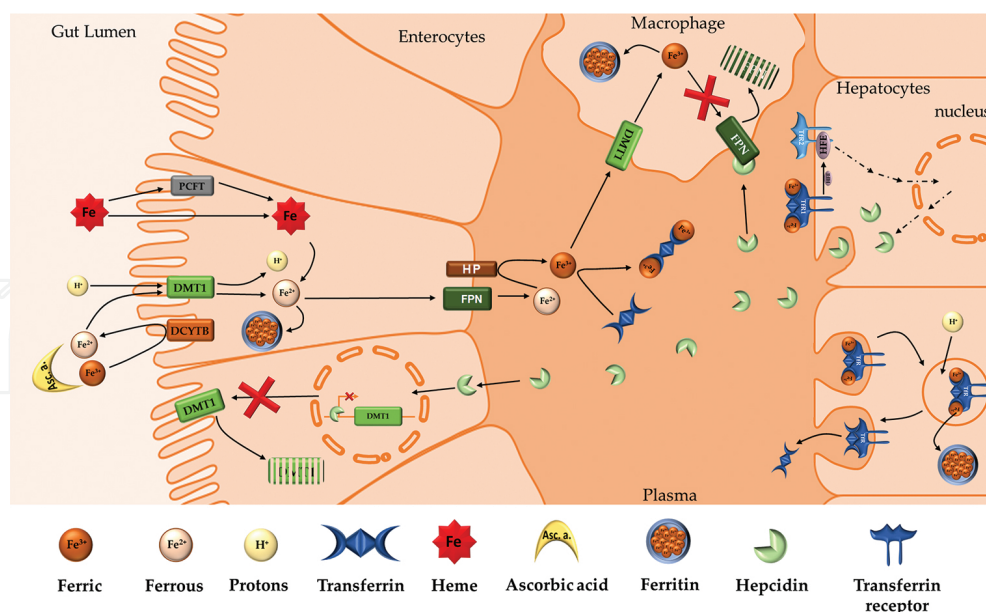
Polyphenols such as tannic and chlorogenic acids are phytochemicals and are mostly present in tea, coffee, red wine, vegetables, fruits, and herbs [100, 101]. These compounds can act as anti-nutrients and inhibit iron absorption into the enterocytes. On the other hand, they have antioxidant properties useful for the human body [102].

Thus, meals containing legumes or whole grains may prevent the proper iron absorption [103]. Therefore, it is necessary to include aliments containing iron-absorption enhancers to a meal. Among these pro-nutrients are plant compounds such as ascorbic acid (vitamin C) and  $\beta$ -carotene (pro vitamin A), but also muscle tissue [104, 105]. Ascorbic acid acts as a reduction factor as well as an iron-chelator facilitating iron transport [106, 107]. It was shown that including  $\beta$ -carotene to the meal improved the iron absorption up to three fold. This effect was observed even if inhibitor-containing food was incorporated [105].

Humans take up heme and nonheme Fe (**Figure 4**). Although absorption of heme iron has not been well described, we know that heme is probably able to cross the lipid bilayer of the cells, but it might also be absorbed via endocytosis as an entire porphyrin structure [108, 109]. Furthermore, a heme carrier protein 1 (HCP1) was described as a mediator for heme transport localized on the enterocytes [110]. However, it was shown one year later that this protein just transports heme incidentally and the actual function is the transport of folate (proton-coupled folate transporter, PCFT). The authors thus named this protein PCFT/HCP1 [111]. Once heme enters the cytoplasm, heme-oxygenases degrade it to release ferrous iron [109, 112], which then binds to ferritin for storage. Since humans do not have an excretion pathway for iron, its excess bound to ferritin is eliminated through the gastrointestinal tract [112].

The iron absorption is triggered when the body-iron sources diminish due to bleeding, inflammation, anemia, or hypoxia. As explained before, nonheme iron from plant food is





**Figure 4.** Iron absorption in humans. Fe from the meal exists in form of heme Fe or free  $\text{Fe}^{3+}$ . Heme is transported via the HCP1/PCFT, and probably absorbed by endocytosis or passing the lipid bilayer.  $\text{Fe}^{3+}$  from plant sources is first reduced by DCYTB. Ascorbic acid enhanced the reduction of iron in the lumen when taken together with meals. Divalent metal transporters such as DMT1 or NRAMP2 take up the reduced Fe into the cells. Fe is stored in form of ferritin or FPN further transports it into the blood plasma. There,  $\text{Fe}^{2+}$  is oxidized back to  $\text{Fe}^{3+}$ , which is either taken up by DMT1 transporter from macrophages or trapped by Tf for the circulation with the plasma. Cells with iron necessity sense and bind the loaded Tf (holotransferrin) with the TfR, which is internalized via endocytosis. Acidification of the vesicles causes the release of Fe, which binds ferritin and the empty TfR is recycled back to the plasma membrane. Iron sufficiency in the cells promotes the complexation of HFE with TfR1 and blocks further binding of holotransferrin. HFE translocate to TfR2 and starts the signal cascade for the production of hepcidin. Hepcidin inhibits the transcription of DMT1 and triggers its internalization and degradation. On the macrophages, hepcidin causes the internalization and degradation of FPN as well.

present as ferric iron ( $\text{Fe}^{3+}$ ). Prior transport, the ingested iron is reduced to ferrous iron ( $\text{Fe}^{2+}$ ) by iron reductase duodenal cytochrome b (DCYTB) which is able to reduce Cu as well [113]. When food rich in vitamin C is included to the meal, the ascorbic acid supports the reduction of iron [107]. The divalent metal transporter 1 (DMT1 or NRAMP2) is responsible for the uptake of  $\text{Fe}^{2+}$  as well as other divalent ions such as Mn, Co, Cu, Zn, and Cd and protons into the enterocytes [114]. Once in the cytoplasm,  $\text{Fe}^{2+}$  is bound to ferritin for storage or if, necessary transported to the plasma via basolateral transporter ferroportin (FPN). Hephaestin (HP), a multicopper ferroxidase, oxidizes  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  allowing its binding to transferrin (Tf) and the circulation in the plasma [115, 116]. This carrier owns two metal-binding sites found on the N- and C-terminal parts of the molecule. Specific Fe-Tf membrane receptors (TfR) recognize the holotransferrin and the Tf/TfR complex is internalized within the cells via clathrin-coated pits endocytosis. The iron is released from Tf through acidification of the vesicles by a proton pump and can be either stored as ferritin or used to cover the needs of the cell. Finally, the empty Tf/TfR complex moves to the cell surface where the apotransferrin is released to the plasma and charged with new iron [117, 118]. Under levels of iron sufficiency, the protein HFE (hereditary hemochromatosis protein) complexes with the TfR1 blocking the binding of transferrin and iron uptake into the cell. By increased holotransferrin concentration in the plasma, it com-

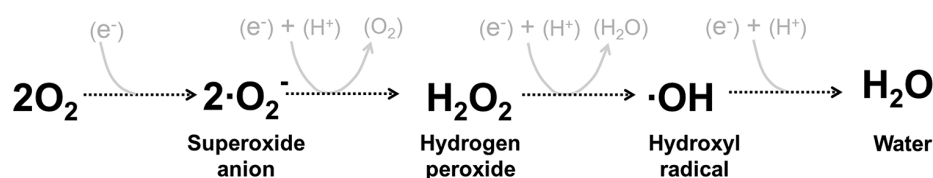
petes with HFE causing its dissociation. HFE then translocate to TfR2 and “inform” the cell about the elevated Fe-Tf status. This HFE–TfR2 complex starts a signal cascade that regulates the transcription of hepcidin [119–121]. The level of hepcidin in blood modulates the signal for the enterocytes to transport iron into the plasma. Hepcidin is a peptide hormone produced in the liver and negatively regulated during inflammation, anemia, and hypoxia [122, 123]. Under conditions of iron sufficiency, hepatocytes secrete hepcidin to block iron absorption of the enterocytes and transport from macrophages. It was shown that hepcidin inhibits DMT1 transcription and promotes protein internalization followed by its degradation [124, 125]. Besides, hepcidin binds ferroportin (FPN) from macrophages causing its internalization and degradation [126–128]. Hepcidin regulation is still unclear, but there is evidence that the transferrin receptor (TfR), matriptase-2 and hemojuvelin increase the hepcidin expression level [129–131]. Due to the high number of proteins involved in Fe absorption, it is not surprising that several human genetic diseases result in an enhanced Fe absorption, leading to the different types of disorders called hereditary hemochromatosis (HH). The reasons for these disorders are mutations in genes involved in the hepcidin–ferroportin signal transduction such as *HFE*, hepcidin gene *HAMP*, *TfR2*, and ferroportin *FPN*. These mutations disturb the hepcidin-mediated downregulation of ferroportin, the iron transporter responsible for the iron load into the plasma. Furthermore, mutations in genes coding for proteins involved in the iron transport cause insufficient supply of iron for heme synthesis. The consequence is anemia and downregulation of hepcidin despite iron overload [121, 132].

## 4. Oxidative stress

### 4.1. Reactive oxygen species (ROS)

The chemical transformation of vital substances in the normal metabolism creates free radicals, which can be any chemical species with one or more unpaired electrons. The free radicals include the hydrogen atom, as the simplest radical, most transition metals and the oxygen molecule. Oxygen radicals, the so-called reactive oxygen species (ROS), are the most common radicals produced in an organism. Among these, the most relevant are the superoxide ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\cdot\text{OH}$ ) [133, 134]. These radicals are produced under normal physiological, pathological, or stress conditions by specific reductase enzymes or in biochemical processes that involve an electron transport chain, for example, photosynthesis, respiration, or oxidative phosphorylation [135]. **Figure 5** shows the complete reduction from oxygen to water with the intermediate radicals. The first step is the reduction of molecular oxygen ( $\text{O}_2$ ) to the relatively stable and reactive superoxide anion ( $\text{O}_2^-$ ). In physiological conditions,  $\text{O}_2^-$  undergoes spontaneously or enzymatically a dismutation reaction and forms hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), which then gives rise to the most reactive oxidant, the hydroxyl radical ( $\cdot\text{OH}$ ) [133, 136, 137].

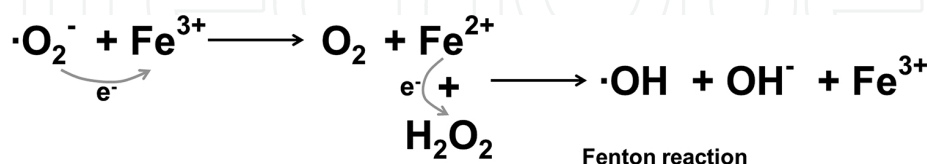
Cells use ROS as messenger molecules for specific responses. For example,  $\text{H}_2\text{O}_2$  plays an important role in the signal transduction for the immune response of both humans and



**Figure 5.** Reactive oxygen species. During processes such as photosynthesis, respiration, and oxidative phosphorylation electrons are transferred to oxygen for the full reduction to water. This process generates very reactive precursors. The first reduction of oxygen generates superoxide anions. Through a dismutation reaction, these anions are converted to form oxygen and hydrogen peroxide. Under the influence of metals, hydrogen peroxide is decomposed and forms the reactive hydroxyl radicals. Dashed arrows and brackets indicate that chemical reactions are not represented in stoichiometrical manner.

plants [138–141]. In humans, it induces the nuclear presence and the DNA-binding of the transcription factor NF- $\kappa$ B, which activates the transcription of genes involved in inflammatory and immune responses [138]. In plants, the immune response is also supported by the production of ROS. These molecules cross-link cell wall proteins to prevent pathogen entrance and induce the production of phytoalexins, which are small molecules that accumulate in the area of infection and prevent growth and spread of the bacteria. Likewise, ROS promote the induction of the hypersensitive response (HR), so that cells undergo programmed cell death to remove nutrient sources for the pathogen. ROS waves are also involved in systemic resistance responses [139, 142].

On the other hand, high production of ROS leads to the so-called oxidative stress. The stimulus of an organism to unaccustomed environments or situations is called stress. The response to such stress might vary from defense and survival to cell death. Toxins, drugs, pollution, and transition metals, iron, in particular, promote the formation of ROS and are thus called prooxidants. When Fe exists abundant in a free state, it is able to catalyze the production of hydroxyl radicals in a two steps reaction called the Haber–Weiss reaction (**Figure 6**).  $Fe^{3+}$  first oxidizes the superoxide anion to oxygen and is reduced itself to  $Fe^{2+}$ . Then,  $Fe^{2+}$  is oxidized in a Fenton reaction, which catalyzes the split of  $H_2O_2$  and the formation of a hydroxyl radical and a hydroxide ion [143–145].



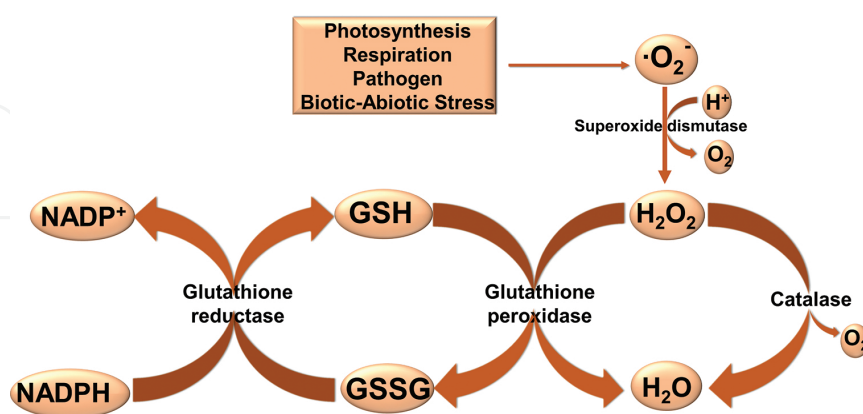
**Figure 6.** Haber–Weiss reaction.  $Fe^{3+}$  catalyzes the production of hydroxyl radicals. Free  $Fe^{3+}$  is reduced to  $Fe^{2+}$  and at the same time oxidizes superoxide anions to oxygen.  $Fe^{2+}$  is oxidized and simultaneously cleaves hydrogen peroxide to a hydroxyl radical and a hydroxide ion, which can in turn further react with many molecules.

Ionizing radiation, inflammation, high chemical concentrations, and cellular conditions determine the production of free oxygen radicals. In the case of a chronic inflammation, an overproduction can damage tissues [146].

As mentioned before, Fe uptake in humans and plants is regulated in a way that over-accumulation does not occur in normal cases, except in the iron overload diseases. Regardless, the reason of an iron accumulation, radicals then generate an endless number of diseases such as hepatic cirrhosis, primary liver cancer, diabetes mellitus, arthropathy, cardiomyopathy, chronic fatigue, joint pain, impotence, and osteoporosis [147]. In plants, ROS accumulate upon drought, salt stress, cold, heat, heavy metals, high light, ozone, mechanical stress, nutrient deprivation, and pathogen attack. Since plants are not able to escape stresses, they developed mechanisms to adapt and in case of stress-induced ROS to avoid the production of further ROS. In high light conditions, for example, plant diminishes the leaf surface by curling, or upon drought, they close the stomata. If the plant is not able to counteract the production of ROS during the mentioned biotic and abiotic stresses, the cells undergo programmed cell death and the plant may die [148].

#### 4.2. Antioxidants and ROS-scavenging

Low molecular weight molecules and enzymatic proteins are responsible for keeping the balance between harmful and useful ROS. Most ROS are produced in the mitochondria, peroxisomes and, in plants, in chloroplasts as well [148, 149]. Hence, the majority of the ROS-scavenging enzymes are located there [150]. The most important enzymes for ROS-scavenging are the superoxide dismutases (SOD) which catalyze the dismutation of superoxide to form hydrogen peroxide and molecular oxygen. Depending on the compartment, the metal in the active site of this enzyme might change. The MnSOD are present in the mitochondrial matrix, FeSOD in the chloroplast, and CuZnSOD in the rest of the cell [151–153]. The hydrogen peroxide is further processed to water and oxygen by a glutathione peroxidase reaction. Glutathione (GSH) is a tripeptide consisting of glutamic acid, cysteine, and glycine. GSH plays a very important antioxidative role because it is produced in high amount in almost all cells in plants and mammals. It has the capacity to easily oxidize to glutathione disulfide (GSSG). Subsequently, the glutathione reductase converts the GSSG back to GSH. Additionally,



**Figure 7.** ROS-Scavenging. SOD are responsible for the dismutation of the reactive superoxide to oxygen and hydrogen peroxide. The glutathione reductase reduces  $\text{NADP}^+$  to NADPH and oxidizes GSH to GSSG. The last served as a substrate for the glutathione peroxidase which decomposes hydrogen peroxide to water and oxygen. Biochemical reactions are not represented in stoichiometrical manner.

catalase is a very efficient enzyme to metabolize the hydrogen peroxide to water and oxygen (Figure 7) [135, 148, 154, 155].

Another important antioxidant is ascorbic acid (vitamin C) which can react with hydroxyl radicals, superoxide, and oxygen. All animals, except primates and guinea pigs, are able to produce ascorbic acid, and in plants, it accumulates in large amounts. In addition, ascorbic acid reduces prolin and  $\alpha$ -tocopherol (Vitamin E) which are important antioxidants as well [156]. In plants, prolin acts as an osmotic agent under salinity and drought. It reacts with hydroxyl radicals to form hydroxyprolin and water, protecting the plant against the radicals [157, 158]. The production of ROS is one mechanism to protect the organism against pathogens attack. The following section will give an overview about the principal mechanisms of pathogen defense in humans and plants.

5. Pathogen defense

The principles of immune responses in plants and mammals differ in several aspects. Mammals have a circulatory system with specialized killing and memory cells as a part of the very effective adaptive immunity. Plants rely on an effective innate immunity.

Plant PRR	Bacterial Antigen	Human PRR
n.d.	Bacterial lipoprotein	TLR 1
LYM1	Bacterial peptidoglycans	TLR 2
LYM3		
CERK1		
n.d.		
n.d.	Double stranded RNA	TLR 3
LORE	Lipopolysaccharides	TLR 4
FSL2	Bacterial flagella	TLR 5
n.d.	Bacterial lipoprotein	TLR 6
n.d.	Viral single strand RNA	TLR 7/8
n.d.	Bacterial viral DNA	TLR 9
n.d.	n.d.	TLR 10
n.d.	Profilin	TLR 11

n.d. = not determined, PRR = Pattern recognition receptors, TLR = Toll-like receptor. References can be found in the text

Table 1. Bacterial PAMPs and the corresponding PRRs from plants and humans.

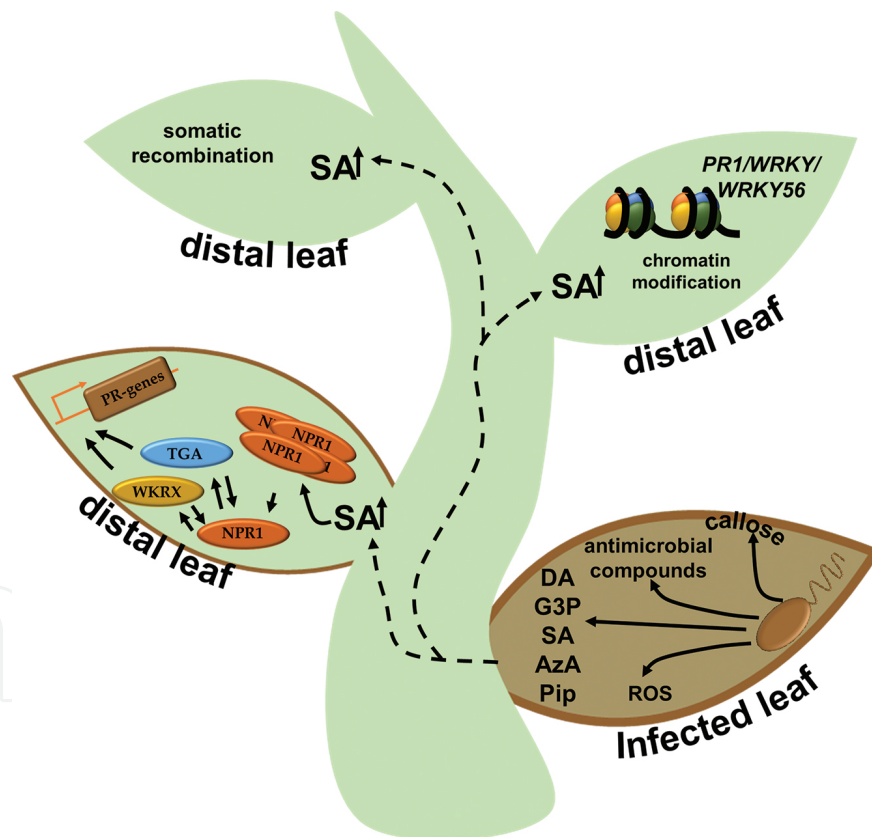
The first protection against pathogens is generally to prevent pathogen entrance into the organisms using physical barriers. In plants, callose, lignin, and other phenolics reinforce the cell walls of wood and xylem vessels after pathogen attack or mechanical damage thereby



preventing the spread of the pathogen and its toxins [159–162]. There are many barriers, which serve for the first pathogen defense in humans. For example, the skin responds with epithelial peels, drying out, and changes of the pH [163]. Eyes and the respiratory tract produce fluids to wash or catch pathogen invaders [164, 165]. If these barriers fail, the innate immune system comes into place, which is present in all animals and plants.

The first immediate (basal) immune defense in plants and animals is the innate immunity response. Specialized pattern recognition receptors (PRR) recognize microbe molecules, which are called microbe-associated or pathogen-associated molecular patterns (MAMPs/PAMPs) [166, 167]. The different PRRs recognize many different PAMPs including chitin, lipopolysaccharides, peptidoglycans, and flagellin [166–170], and interestingly, similar patterns are recognized by receptors and proteins in plants and animals (**Table 1**).

In plants, the best-characterized receptor is called FLAGELLIN-SENSING 2 (FLS2) and in *Arabidopsis* was found to recognize the bacterial flagellin epitope (fls22), a 22-amino acid peptide. FLS2 triggers the immune responses, including cell wall fortification and ROS production, and its signaling cascade has been well characterized in the last years [166, 171–



**Figure 8.** SAR and priming. The attack of a pathogen leads to signals, which give to the plant an immediately protection and a long-lasting immunity. The infected leaf generates callose for the fortification of cell walls, ROS and antimicrobial compounds to kill the bacteria, and defense-related metabolism. These processes are part of the innate immunity. Principally, the metabolite SA is transport through the phloem to the other part of the plants activating the SAR, which is a preventive defense mechanism. This includes the monomerization of NPR1 that in turn activates PR-genes, chromatin modification such as methylation and acetylation of histones and somatic recombination.

173]. The immune response triggered by MAMPs/PAMPs is called PTI (PAMP-triggered immunity). Some bacteria developed a mechanism to bypass the plant pathogen response by injecting effectors (virulence proteins) into plant cells via the bacterial type III secretion system (TTSS). These effectors in turn may be recognized by leucine-rich repeat (NBS-LRR)-type plant proteins and receptors. These resistance factors trigger a second immune response, the effector-triggered immunity (ETI) [174], which may also result in the hypersensitive response (HR), systemic acquired resistance (SAR) including a transcriptional activation of PR proteins, as well as salicylic acid hormone signaling [175–177].

The basic immune output responses are important mechanisms for the surviving of the plant during and after pathogen attack. The HR is an emergency measure, which activates cell death (programmed cell death, PCD) of a limited number of surrounding cells to limit the nutrients and thereby avoid the spread of biotrophic pathogens [178]. Moreover, ETI and PTI activate the so-called systemic acquired resistance (SAR) and the corresponding pathogenesis-related (PR) genes. SAR is a long-distance and long-lasting immune response activated in the entire plant after a local infection [179, 180]. **Figure 8** shows an overview of the forwarding signal of the innate to the acquired immunity.

Fourteen known classes of PR-genes (PR1–PR14) [181] are coordinately expressed to counter-attack the pathogens. These genes encode hydrolytic enzymes such as  $\beta$ -1,3-glucanase, chitinase, and plant defensins, which hydrolyze pathogen cell walls and disrupt pathogen membranes [182]. Additionally, the PR-genes encode enzymes needed for the synthesis of callose for the fortification of barriers, and defense-related metabolites such as salicylic acid (SA), diterpenoid dehydroabietinal (DA), a glycerol-3-phosphate (G3P)-dependent factor, azelaic acid (AzA), and pipecolic acid (Pip) [183]. These metabolites are loaded in the phloem and transported to uninfected parts of the plants and “warn” them of an attack [182]. In the distal parts, SA causes the monomerization of the NONEXPRESSOR OF PR GENES 1 (NPR1), allowing its transport to the nucleus. Following, it interacts with TGACG motif-binding protein (TGA) and WRKY transcription factors for the activation of many PR-genes [183–185]. SA accumulation upon pathogen attack and treatment with the protector molecule  $\beta$ -aminobutyric acid (BABA) also provides the plant with a “memory,” which allows cells to respond faster and stronger to a secondary challenge. This process is called priming [186, 187]. It was shown that after treatment with a synthetic analog of SA transcript and protein of the MITOGEN-ACTIVATED PROTEIN KINASES 3 AND 6 (MPK3, MPK6) accumulated in upper leaves after infection of lower leaves [188]. It was reported that priming after a certain attack can be mediated through somatic recombination and modification of the chromatin such as acetylation and methylation of histones [182, 189–191] (**Figure 8**).

Humans have an innate immune system as well, which is the first response to a pathogen attack. The innate immune system provides the organism with a rapid but not specific response. Surface barriers like skin, fluids and antimicrobial peptides prevent the entrance of the pathogens into the body [163]. When pathogens pass these barriers, the body attempts to eliminate the source of injury and damaged tissue. This is carried out by the generation of an inflammation, which includes high blood irrigation, immune cells, and mediators. Inflammation is the first immune reaction to a pathogen invasion or injury and corresponds to the innate

immune response, which works in a similar way as in plants [192]. Bacterial components, best known as PAMPs (**Table 1**), are recognized by PRR. They are called in humans toll-like receptors (TLR1-11). These receptors are on the surface of mast cells, macrophages, and dendritic cells [193, 194]. Once a mast cell recognizes a PAMP, it secretes mediating factors, such as histamine or tumor necrosis factor (TNF $\alpha$ ). These factors dilate the blood vessels enabling neutrophils to enter the damaged tissue. Bacteria-degrading substances are secreted, and bacteria are removed by phagocytosis [194]. The activated macrophages phagocytose bacteria produce cytokines and prostaglandins, which attract neutrophils, monocytes, and dendritic cells. Cytokines are responsible and induce a rise of the body temperature (fever) because the growth of many pathogens is then compromised. Dendritic cells identify PAMPs and mature to antigen presenting cells (APC). They process the antigens and recruit them to the T-lymphocytes to start the adaptive immune answer. Hence, the dendritic cells are the link between the innate and the adaptive immune system [195].

### 5.1. Iron and pathogen defense

Pathogens gain from the host all required nutrients. Iron regulation is very important for pathogen survival during infection in plants. Iron plays dual roles for host and pathogen, either as nutrient or as essential cofactor constituent to initiate or avoid immune responses.

The genes *FER2* and *FER1* of a maize fungi *Ustilago maydis*, which encode a high-affinity iron permease and an iron multicopper oxidase respectively, are involved in the iron uptake in this microorganism. Deletion of these genes showed that the infection rate of the fungi in maize plants was impaired, concluding that these genes are crucial for its virulence as well [196]. Furthermore, NPS6 is a virulent gene conserved in many filamentous fungi, which is involved in siderophore biosynthesis and in tolerance to H<sub>2</sub>O<sub>2</sub> [197]. The requirement to sequester iron via siderophore production might be not only to take up iron but also to protect from reactive oxygen species [198].

In the host cells, iron exists mostly in complex with ferritin, transferrin, hemoglobin, and other proteins. It was shown that after a pathogen infection in plants ferritin accumulates, possibly to protect from ROS but also to deprive invaders from iron [199]. During a pathogen attack, iron accumulates in the apoplast to elevate the oxidative response, which in turn activates the expression of PR-genes. The translocation of iron to the apoplast causes intracellular iron deficiency activating iron uptake genes and PR-genes [200]. In mammals, during an infection or inflammation the hepcidin level rises, this stops the iron absorption and stimulates the transport of circulating iron (or free heme from damaged tissue) into the macrophages. This is a mechanism controlled by cytokines [201]. However, many pathogens developed the property to gain iron from transferrin and heme. For example, *V. cholerae* is able to induce the release of heme from hemoglobin. *Hemophilus influenzae* or *Trichomonas vaginalis* trigger release of iron from transferrin [202]. Bacteria stimulate siderophore production, which may serve to solubilize iron inside the host and transport it into the microbial cells [16, 203]. The upregulation of ferritin may help to withstand siderophore action [199].

However, many bacteria can also act in a positive way on plants. In the plant rhizosphere, many beneficial nonpathogenic rhizobacteria are present, which protect the plants against

pathogenic microorganisms by secreting antimicrobial components [204]. The content of plant growth-promoting rhizobacteria (PGPR), soil type, and strategy of iron uptake plays a fundamental role for plants in the iron nutrition [205, 206]. In turn, the iron status of the plant influences the rhizobacteria community as well. Studies in barley and tomato showed that the bacterial community in rhizosphere of iron deficient plants is much smaller than of plants grown in iron sufficient conditions. Additionally, the different iron uptake strategies of these two plants leads to a different qualitative and quantitative patterns of the rhizobacterial population [206].

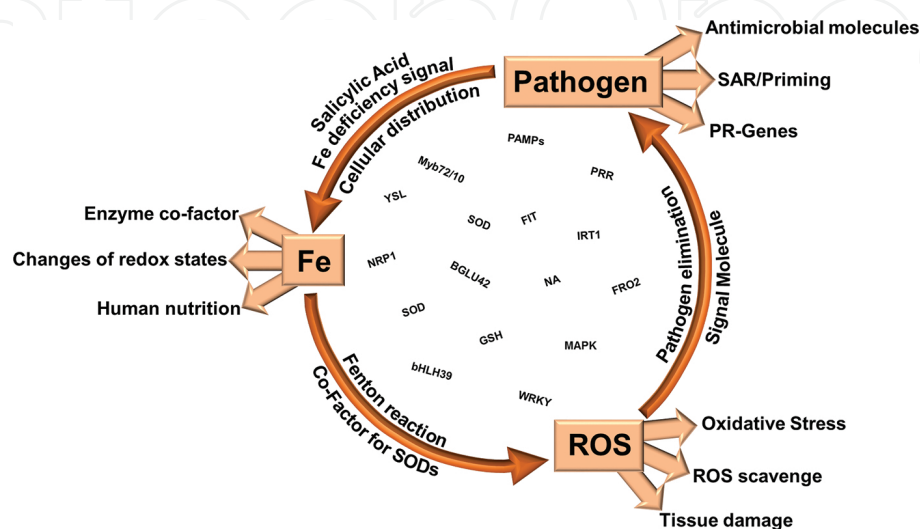
Plants may profit from siderophore production of the rhizobacteria for the activation of the induced systemic resistance (ISR), a SA-independent immunological pathogen response in plants [207, 208]. Iron deficiency and ISR are closely related. Transcriptome analysis in *A. thaliana* showed that a high number of genes upregulated upon iron deficiency conditions are also upregulated in plants treated with beneficial bacteria [209]. Similarly, treatment with synthetic siderophores upregulated many genes encoding for WRKY transcription factors and genes required for the iron uptake, such as ferritin, the iron transporter (IRT1), the iron reductase (FRO2), and the NICOTIANAMINE SYNTHASE (NAS) [199, 210]. An overlap between these two processes is represented by the transcription factor MYB72. This transcription factor activates downstream an important component necessary for ISR called BGLU42 ( $\beta$ -glucosidase). BGLU42 is involved in the production and secretion of phenolic compounds from the root to the rhizosphere and is a key component for the activation of the ISR [87]. MYB72 is strongly upregulated under iron deficiency conditions, and its regulation occurs in a FIT-dependent manner [85, 86]. Thus, mutants lacking MYB72, and its close homologue MYB10, were not able to survive in iron deficiency conditions. Moreover, it was shown that MYB72 induces the expression of *NAS4* [51, 85] and *BHLH039* [87].

The effect of siderophores on Fe homeostasis is comparable with treatments with BABA. BABA ( $\beta$ -aminobutyric acid) is a nonprotein amino acid priming the plants against a broad-spectrum of pathogens as well as abiotic stresses such as salt stress and drought. The mechanism of protection is based on the activation of the SA-dependent pathogen response, abscisic acid (ABA)-dependent formation of callose [211–213]. Moreover, BABA affects the Fe-homeostasis upon reducing ferritin and increasing *NAS4* transcript and protein. Plants treated with BABA show similar phenotype, transcription of *IRT1* and *FRO2* and metabolite composition as iron deficient plants. BABA is able to chelate iron and mimic the effect of the pathogen siderophores intensifying the theory that iron scavenging is a strategy for the activation of pathogen responses [210, 214].

An additional overlap between Fe homeostasis and pathogen defense is provided by the plant hormones SA. As mentioned before, SA is necessary for the activation of PR-genes and the priming processes for the long-lasting defense [182, 183, 187]. The transcription factor OBP3 (OBF-BINDING PROTEIN 3) is induced by SA and is involved in plant growth and development [215, 216]. Studies in OBP3-overexpression lines had shown that *BHLH038* and *BHLH039* were strongly upregulated [217]. However, an effect of SA on iron deficiency response regulation has later not been found in the wild type or in the triple mutant *bhlh039 bhlh100 bhlh101* [78].



**Figure 9** shows the relationship of Fe homeostasis with the production of ROS and the pathogen response. Fe is required as a cofactor for the production of ROS. The local iron deficiency induced by bacterial siderophores upregulates *MYB72*, which in turn activates the expression of *BHLH039*, *NAS4*, and *BGLU42*. Additional observations have also led to the conclusion that pathogens and the hosts may compete for Fe and that Fe may be required, at the same time, for inducing defense but also to sustain pathogen infection [207, 208].



**Figure 9.** Correlation between Fe, ROS, and pathogen defense. All three processes are tightly related. Besides the initiation of pathogen responses during microorganism attack, Fe translocates to the apoplast causing a local Fe deficiency and increasing Fe uptake from the roots. High levels of Fe lead to the accumulation of ROS, but simultaneously it serves as a co-factor for enzymes involved in the ROS scavenging. ROS molecules support the elimination of pathogens and activate local PR genes. In the center of the ring are represented the genes involved in the three processes.

## 6. Breeding for better crops

Selection of better plants, genetic engineering, and breeding can also be used for the generation of nutrient-rich crops. A good example is provided by the rice IR68144 line, which presents tolerance against diseases, high yields, and high Fe and Zn content [218]. Studies in Philippine women showed that the consumption of this rice versus the normal diet increased the iron content in the body [219]. Nowadays, the world population grows with great rapidity and the use of common breeding methods may not be sufficient to cover the necessity of better crops and yield. It is possible to increase the iron content of edible organs of plants by improving iron mobilization in the soil and plant, the storage and remobilization in the leaves, grains or fruits.

In rice, the insertion of phytosiderophore synthesis genes from barley increased considerably its tolerance to calcareous soil and Fe and Zn content in the grain [220]. The introduction of a yeast Fe reductase into rice increased likewise yield and the tolerance to a Fe deficiency environment. However, the iron content of the grain was not improved [221]. The *A. thaliana*



IRT1 transports not only Fe but also other divalent metal cations such as Zn, Mn, and Co [222]. Increased activity of Fe transporters may lead partially to an increased iron content in the grain but simultaneously may also compromise plant health due to the accumulation of other metals, which in turn leads to a high ROS production [223]. Furthermore, it is important that in addition to an increased iron content in the plants, attention should also be paid to iron bioavailability for humans.

Seeds have a high amount of phytic acids, which form a complex with iron and inhibit its absorption in the intestinal lumen [97]. Maize plants co-expressing the phytic acid-degrading enzyme phytase from *Aspergillus niger* and ferritin from soybean under the control of an endosperm promoter, increased up to threefold the iron bioavailability in *in vitro* experiment with Caco-2 cells [224]. Similar in rice, the expression of ferritin from *Phaseolus vulgaris*, phytase from *Aspergillus fumigates*, and the endogenous cysteine-rich metallothionein-like protein improved iron bioavailability for humans [225]. Genetic approaches in *Lactuca sativa* (common Lettuce) showed that introducing the soybean ferritin gene is sufficient to increase the iron content in the leaves, boost the growth rate, and enhance the size up to 40% [226].

The pathogen defense of plants can be also improved using genetic methods. PR-genes involved in the production of antimicrobial substances such as chitinases,  $\beta$ -1,3-glucanase, defensins, osmotin, and phytoalexins can be used to increase the strength of crops against biotic stresses [227]. Osmotin is a PR-gene from tobacco expressed under biotic and abiotic stresses, which causes holes in the plasma membrane of several fungi [228, 229]. Overexpression of osmotin in potato leads to a delay of the disease caused by *Hytophthora infestans* [230]. Defensins are cysteine-rich peptide with antimicrobial properties in humans and plants [231, 232]. When expressed in potato, the antifungal protein (AFP) from *Medicago sativa* seeds leads to strong resistance against the fungal pathogen *Verticillium dahlia* [233]. The fungal cell wall is a very complex structure composed of many different proteins as well as chitin and glycan, which are essential components [234]. Chitinases and glucanases are potent antifungal enzymes and hydrolyze fungal cell wall [227]. Apple trees transformed with the endochitinase from *Trichoderma harzianum* present an important reduction in the number of lesions and its area after *Venturia inaequalis* inoculation [235].  $\beta$ -1,3-glucanase inhibits the grow of many fungi as in the case of flax (*Linum usitatissimum* L.). Several transgenic lines caring the potato  $\beta$ -1,3-glucanase present high resistance against *Fusarium* species, demonstrating that degradation of fungal cell walls is a weapon against fungi [236].

Many abiotic factors are causing oxidative stress in plants. Investigation on the aluminum-induced genes showed that Arabidopsis plants of the ecotype Ler-0 expressing the blue-copper-binding protein (AtBCB), the peroxidase gene (AtPox), or the tobacco glutathione S-transferase gene (parB) were more resistant to treatment with oxidative stress inducing diamide [237]. The overexpression of CuZn-Superoxid dismutase (CuZnSOD), Mn-Superoxid dismutase (MnSOD), and ascorbate peroxidase (APX) in transgenic tobacco plants were more tolerant to the viologen (MV, paraquat)-mediated oxidative damage [238]. Ascorbic acid is an important antioxidant [156] in plants. Tomato (*Solanum lycopersicum*) plants overexpressing GDP-Mannose 3',5'-epimerase (*SIGME1* and *SIGME2*) are able to accumulate

ascorbic acid and improved the tolerance to viologen stress, cold stress, and better biomass under salt stress.

To date, there are only a few examples of how the genetic engineering is able to improve plant growth and crop yield during situations of pathogen attack and different abiotic stresses such as nutrient deficiency or environmental stresses.

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