Ferroptosis in plants: triggers, proposed mechanisms and the role of iron in modulating cell death

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Highlight

This review summarizes recent progress in our understanding of plant ferroptosis, focusing on environment-responsive signaling pathways and underlying mechanisms.

Abstract

Regulated cell death plays key roles during essential processes along the plant life cycle. It takes part of specific developmental programs and maintains the organism homeostasis in response to unfavorable environments. Ferroptosis is a recently discovered iron-dependent cell death pathway characterized by the accumulation of lipid reactive oxygen species. Ferroptosis in plants shares all the main hallmarks described for ferroptosis in other systems. Those specific features include biochemical and morphological signatures that seem conserved among species. However, plant cells have specific metabolic pathways and a high degree of metabolic compartmentalization. Together with their particular morphology, these features add more complexity to the plant ferroptosis pathway. In this review, we summarize the most recent advances in elucidating the roles of ferroptosis in plants, focusing on specific triggers, main players and underlying pathways.

Key words: Cell death, ferroptosis, iron, lipid peroxidation, heat stress, biotic stress

Introduction: Cell death in plants

Regulated cell death (RCD) is an active and controlled process that allows organisms to selectively eliminate specific cells in a highly coordinated fashion through the activation of molecular mechanisms and pathways. In plants, regulated cell death plays essential roles during the plant life cycle, driving specific gametophytic and sporophytic developmental programs. Some examples include the differentiation of tracheary elements, suspensor degeneration during embryogenesis or remodeling of leaf shape (Gunawardena, 2008; Courtois-Moreau *et al.*, 2009; Drews and Koltunow, 2011; Bollhöner *et al.*, 2012; Choi, 2013; Xie *et al.*, 2014). Regulated cell death is also considered as a mechanism that allows plants to maintain homeostasis under unfavorable environments or in response to a critical stress. As damaged tissues are removed, the stress signal is systemically amplified, resulting in plant survival. Diverse environmental stimuli are able to trigger RCD: salinity, drought, UV radiation, heavy metals and extreme temperatures are among the abiotic stresses known to activate cell death mechanisms (Barnabás *et al.*, 2008; Liu *et al.*, 2009; Petrov *et al.*, 2015; Distéfano *et al.*, 2017; Srivastava *et al.*, 2018; Chua *et al.*, 2019).

Cell death pathways are also crucial to protect plants from pathogens. A localized cell death, the hypersensitive response (HR), is essential to trigger a systemic response and to limit infection spreading (Kiba *et al.*, 2006; Coll *et al.*, 2010; García-Marcos *et al.*, 2013; Dangol *et al.*, 2019; Noman *et al.*, 2020).

Although RCD can be triggered by a variety of developmental and environmental stimuli, reactive oxygen species (ROS) have been proven to play a central role in these cell death pathways (Doyle *et al.*, 2010; Xie *et al.*, 2014; Petrov *et al.*, 2015; Liu *et al.*, 2018; Cai *et al.*, 2020). ROS are produced in different cell compartments. However, chloroplasts and mitochondria are among the principal organelles that contribute to the pool of ROS produced during RCD (Liu *et al.*, 2007, 2014; Cvetkovska and Vanlerberghe, 2012; Bi *et al.*, 2014; Wu *et al.*, 2015). The concentration of ROS affects in turn not only the organelles' redox-status but also the redox state of antioxidants such as plastoquinone, ascorbate, and glutathione pools (Foyer and Noctor, 2011; Noctor *et al.*, 2012).

Plant cells do not display many of the morphological features that distinguish apoptosis (van Doorn *et al.*, 2011). However, their large vacuole plays a key role during a plant-specific type of cell death that involves a gradual decrease in the cytoplasm volume and the formation of small lytic vacuoles (Jones, 2001) . This type of cell death is called vacuolar cell death (VCD) and has been associated with xylem cell death and with the hypersensitive response (Bollhöner *et al.*, 2012; Vorster *et al.*, 2019). VCD involves tonoplast rupture, disassembly of the nuclear envelope and nuclear segmentation, while mitochondria and other plant organelles remain intact until the final stages of this pathway (van Doorn *et al.*, 2011). Notably, autophagy (ATG) related genes activation has also been implicated in VCD (Teper-Bamnolker *et al.*, 2019).

Even when plant RCD shows specific features, several events that characterize plant cell death overlap with processes observed in non-plant species, including mammals and other vertebrates. Among these characteristics ROS accumulation, lipid peroxidation and depletion of antioxidants emerge as key conserved hallmarks that are commonly detected during RCD across evolutionarily distant species.

Ferroptosis: discovery and main players

Ferroptosis is an iron-dependent type of cell death characterized by the accumulation of lipid reactive oxygen species and with unique biological and molecular characteristics. Although cell death pathways involving iron and lipid peroxidation have been observed in diverse species for a long time, the term and concept of ferroptosis was first introduced in 2012 to describe a cell death pathway found in tumor cells (Dixon *et al.*, 2012). Ferroptosis defines a type of cell death that is genetically, morphologically and biochemically distinct from other forms of RCD. It does not show the morphological features that characterize necrosis, such as swelling of the cytoplasm and organelles and rupture of the cell membrane. It also differs from cell apoptosis, as cell shrinkage, chromatin condensation and the formation of apoptotic bodies are all features that are not observed in cells undergoing ferroptosis. Morphologically, ferroptosis manifests with a noticeable shrinkage of mitochondria that also show increased membrane density and fewer

mitochondrial cristae. When ferroptosis was first described, the mechanism involved the action of erastin, a small molecule that inhibits the cystine/glutamate antiporter (system Xc-) in tumor cells limiting the availability of cysteine in the cell. As cysteine is required for glutathione (GSH) synthesis, erastin treatments resulted in GSH depletion. In addition, this was accompanied by an overwhelming accumulation of cytosolic and lipid ROS, that were then postulated as the main triggers of cell death (Dixon *et al.*, 2012). Two years later, glutathione peroxidase 4 (GPX4) was identified as a main player of ferroptosis (Yang *et al.*, 2014; Friedmann Angeli *et al.*, 2014). GPX4 is a phospholipid hydroperoxidase that prevents uncontrolled peroxidation of phospholipids. The accumulation of lipid peroxides is highly dependent on iron availability. Chelation of intracellular iron using deferoxamine (DFO) is sufficient to prevent ferroptotic cell death even in *Gpx4* knockout mice (Friedmann Angeli *et al.*, 2014).

Poly-unsaturated fatty acids (PUFAs) -containing membrane phospholipids are the most common lipids that undergo oxidation during ferroptotic cell death (Yang *et al.*, 2016). Lipidomic studies in animal cells have identified phosphatidylethanolamines containing arachidonic acid (C20:4) or the elongation product, adrenic acid (C22:4), as key phospholipids that are targets of peroxidation upon ferroptosis induction (Kagan *et al.*, 2017). Although the source of iron involved in lipid peroxidation has not been identified, it is thought to come from labile iron pools within the cell. The generation of toxic lipid-peroxides may result from enzymatic or non- enzymatic processes that involve different forms of catalytically active iron. The non-enzymatic mechanism involves Fenton chemistry, which generates highly toxic hydroxyl and peroxyl radicals. Enzyme-dependent processes involve iron-containing enzymes such as lipoxygenases (LOXs) (Stockwell *et al.*, 2017), which have been identified mediating lipid peroxidation as a specific downstream event of GPX4 inactivation in mice (Seiler *et al.*, 2008). Since its discovery, ferroptotic cell death has been described in diverse mammals and non-mammalian vertebrates, invertebrates, (Conrad *et al.*, 2018), fungi (Shen *et al.*, 2020) and plants (Distéfano *et al.*, 2017; Hajdinák *et al.*, 2019; Dangol *et al.*, 2019; Macharia *et al.*, 2020).

Ferroptosis in plants

A few years after the discovery of ferroptosis in animals, this process was also described in plants. Distéfano et al. (2017) found a type of cell death in *Arabidopsis thaliana*, which shared many of the hallmarks described for animal ferroptosis: iron-dependent ROS accumulation, lipid peroxidation and glutathione depletion (Distéfano *et al.*, 2017). In addition, many morphological characteristics of mammal ferroptosis were also observed in plant cells: retracted cytoplasm, normal nuclei and mitochondria shrinkage. Ferroptosis in plants was triggered in roots exposed 10 min to a 55°C heat shock or in seedlings exposed for 1 h at 43°C and was prevented by pre-incubation with the canonical ferroptosis inhibitors Ferrostatin-1 (Fer-1, a lipophilic antioxidant) and Ciclopirox olamine (CPX, an intracellular iron chelator) (Distéfano *et al.*, 2017). This pathway was not observed when plant cells were exposed to higher temperatures (77°C) or to other types of abiotic stresses (such as saline and oxidative stress), which led to a cell death pathway that was not preventable by the use of ferroptosis inhibitors. Furthermore, no evidence on the effect of ferroptosis inhibitors on vascular or reproductive development was found so far (Distéfano *et al.*, 2017).

The oxidative burst observed in Arabidopsis roots upon heat stress is preceded by glutathione and ascorbic acid depletion (Distéfano *et al.*, 2017). Fer-1 or CPX are not able to prevent this, suggesting that such depletion might be an early event in the cell death pathway that follows heat stress and not a consequence of lipid peroxidation (Distéfano *et al.*, 2017). GSH depletion can be explained by its extensively consumption in the ER lumen to repair disulfides formed as a consequence of high temperatures, which is also accompanied by an inactivation of GSH biosynthesis (Ozgur *et al.*, 2014). In the absence of available GSH, ascorbic acid cannot be recycled, which can explain the low levels of reduced ascorbic acid detected in Arabidopsis roots after heat stress (Foyer and Noctor, 2011; Noctor *et al.*, 2012; Distéfano *et al.*, 2017).

In animal systems, the selenoenzyme glutathione peroxidase GPX4 has been identified as a main regulator of ferroptosis (Conrad and Friedmann Angeli, 2015; Conrad *et al.*, 2018). In the presence of GSH as the hydrogen donor, GPXs reduce H_2O_2, organic hydroperoxides and lipid peroxides. GSH depletion causes the inactivation of GPX4, which

results in the accumulation of toxic lipid peroxides. Plant GPXs contain cysteine instead of selenocysteine in their active site, a difference that might explain their lower activities when compared to their mammalian counterparts. This idea is supported by site-directed mutagenesis experiments that showed that a selenocysteine to cysteine substitution decreases the catalytic activity of the mammalian GPX by 2-3 orders of magnitude (Bela *et al.*, 2015). It is also important to note that plant GPXs generally use thioredoxin as a reductant agent instead of glutathione, e.g. AtGPX8 (Gaber *et al.*, 2012). Although several GPX proteins are able to use both glutathione and thioredoxin as electron donors, the thioredoxin regeneration system was shown to be more efficient than the glutathione system (Gaber *et al.*, 2012; Bela *et al.*, 2015; Attacha *et al.*, 2017).

Plant GPXs have important roles alleviating the oxidative stress caused by several stimuli. Plants impaired in AtGPX8 show high levels of membrane phospholipid peroxidation, indicating a physiological role in the defense of Arabidopsis cells against oxidative stress (Gaber *et al.*, 2012). In agreement with this physiological role moderating oxidative stress, GPX proteins were also shown to play key functions during ferroptotic cell death processes. The expression of a tomato GPX encoding gene was sufficient to inhibit cell death induced by salt and heat stress in *Nicotiana tabacum* plants (Chen *et al.*, 2004). In addition, treatment of Arabidopsis cells with the GPX inhibitor RSL3 leads to cell death, which can be prevented by the ferroptosis inhibitors Liproxstatin-1 and Fer-1 (Hajdinák *et al.*, 2019). Moreover, a recent study in *Nicotiana benthamiana* showed that silencing of *NbGPX4* leads to enhanced ferroptotic cell death induced by tobacco mosaic virus 24A+UPD infection. *NbGPX4* shares high sequence similarity to *AtGPX6* (Macharia *et al.*, 2020). Altogether, these data suggest an active role for GPX proteins modulating plant ferroptosis.

Carotenoids are well known antioxidants with recognized activity in both plants and animals. Interestingly, oxidation of β-Carotene by O_2 generates an array of cleavage products of varying chain length that are thought to act as signaling molecules in response to stress conditions (Ramel *et al.*, 2012). In Arabidopsis, exposure to high light induces the oxidative cleavage of β-carotene to β-cyclocitral (β-CC), which in turn induces the transcription of genes that respond to lipid peroxidation stress (D'Alessandro *et al.*, 2018).

Remarkably and supporting a putative role of carotenoids regulating ferroptosis, application of β-Carotene to humans cells was shown to inhibit ferroptosis (Yagoda *et al.*, 2007; Yang and Stockwell, 2016).

Plant ferroptosis is highly dependent on PUFAs peroxidation. Pretreatment with PUFAs containing the heavy hydrogen isotope deuterium at bis-allylic carbons (D-PUFAs), which inhibits PUFA oxidation, prevents heat stress-triggered ferroptosis in root cells (Distéfano *et al.*, 2017). LOXs were reported to mediate ferroptosis in animal cells (Conrad et al., 2018; Kagan et al., 2017) through the specific peroxidation of PUFA-PE. Such substrate specificity seems to be directed by a small scaffolding protein, phosphatidylethanolamine-binding protein 1 (PEBP1), that binds to two LOX isoforms (15LO1 and 15LO2) changing their substrate competence to generate hydroperoxy-PE (Wenzel *et al.*, 2017). The PEBP gene family is present in all eukaryotes. In plants, the PEBP family can be divided into three subfamilies, TERMINAL FLOWER1 (TFL1)-like, FLOWERING LOCUS T (FT)-like, and MOTHER OF FT AND TFL1 (MFT)-like (Wang *et al.*, 2015). TFL1 and FT both control flowering time and plant architecture in *A. thaliana*, while MFT plays a critical role regulating seed germination via the abscisic acid and gibberellic acid signaling pathways (Vishal and Kumar, 2018). However, there are no reports on the involvement of plant-specific PEBPs in the modulation of lipid peroxidation or cell death so far.

Lipid peroxidation triggered by LOX enzymes constitutes a hallmark for the cell death that takes place during specific plant pathogen responses. Arabidopsis LOX isoforms are classified as 9-LOXs or 13-LOXs according to the position at which the oxygen is incorporated into the LOX substrates (Zhao *et al.*, 2014). While LOX1 and LOX5 are 9-LOXs, LOX2, LOX3, LOX4, and LOX6 are 13-LOXs. It has been shown that silencing either 9-LOXs or 13-LOXs attenuated the programmed cell death (PCD)-associated by infection with either Potato Virus X-Potato Virus Y or Tomato Spotted Wilt Virus (PVX-PVY and TSWV respectively, (García-Marcos *et al.*, 2013)). A pepper 9-LOX (CaLOX1) was also shown to be involved in cell death. Transient expression of *CaLOX1* in pepper leaves was sufficient to induce cell death and defense responses (Hwang and Hwang, 2010). In addition, silencing of *CaLOX1* resulted in a reduced expression of defense-related

genes, lower lipid peroxidation, a decrease in ROS and salicylic acid accumulation (Hwang and Hwang, 2010). Interestingly, 9-LOX activity on linoleic acid produces 10-oxo-11-phytoenoic acid (10-OPEA) and a series of related 12- and 14-carbon cyclopente(a)nones, collectively named "death acids" (DAs). DAs mediate defense gene expression and promote cytotoxicity resulting in cell death (Christensen *et al.*, 2015). A lipidomic analysis in the interaction of Arabidopsis with *Pseudomonas syringae* revealed that lipid peroxidation precedes cell death and that is mainly confined to plastid lipids including galactolipid and triacylglyceride species. While singlet oxygen was identified as the major cause of lipid oxidation in control conditions, the activity of LOX2 and free radical-catalyzed lipid oxidation increases significantly upon pathogen infection. Additionally, the analysis of mutants impaired in LOX2 revealed that its activity is essential for enzymatic membrane peroxidation induced by the plant-pathogen interaction (Zoeller *et al.*, 2012). Recently, a study showed that acrolein, a highly reactive α,β-unsaturated aldehyde might mediate ferroptosis in Arabidopsis cell cultures (Hajdinák *et al.*, 2019). The addition of acrolein is sufficient to induce cell death, which is mitigated by canonical ferroptosis inhibitors and GSH (Hajdinák *et al.*, 2019).

Lipid peroxidation can also result from a non-enzymatic pathway. The non-enzymatic catalysis of lipid peroxidation is a consequence of the radicals formed through the Fenton reaction, where iron reacts with endogenously produced hydrogen peroxide to form oxygen radicals ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO$). This oxygen radical is able to abstract hydrogen from an unsaturated fatty acid, to yield a lipid-peroxyl radical (Yang *et al.*, 2016; Gaschler and Stockwell, 2017). This non-enzymatic reaction was suggested to take place during plant-pathogen interactions. It has been shown that Fe^{3+} and ROS accumulate at the site of inoculation in several incompatible plant-pathogen interactions (Dangol *et al.*, 2019). Such co-accumulation indicates that Fe^{3+} might result from the reaction between highly reactive Fe^{2+} and H_2O_2 to produce Fe^{3+} and hydroxyl radicals, which can ultimately result in the iron-dependent accumulation of toxic lipid ROS observed during ferroptosis (Dixon *et al.*, 2012; Distéfano *et al.*, 2017).

Additionally, a search for putative orthologs of ferroptosis markers in *Arabidopsis thaliana* revealed that *Kiss of Death* (*KOD*), a gene encoding for a 25-amino-acid peptide

that induces cell death in Arabidopsis roots, is significantly up-regulated in response to HS in a Fer-1 sensitive manner. This suggests that KOD might act downstream of GSH depletion and ROS accumulation in the cascade of events that lead to HS-induced ferroptosis in plants (Distéfano *et al.*, 2017).

Iron and ferroptosis in plants

Due to its redox properties, iron serves as a cofactor of many important enzymes required for a large range of metabolic processes. It facilitates hydroxylation and (de)hydration reactions like the activation and decomposition of peroxides and the electron transfer via an assortment of electron carriers with different redox potentials. However, due to its high reactivity, it could also lead to oxidative stress. To overcome this dual activity, living organisms have evolved complex mechanisms and pathways that regulate iron homeostasis. Plants and animals are able to sense iron availability and to regulate a plethora of iron transporters, chaperons, scavengers, and iron-containing enzymes (Bogdan *et al.*, 2016; Connorton *et al.*, 2017). As intracellular iron is essential for ferroptotic cell death, all the players involved in the regulation of iron availability and metabolism can also play a key role modulating ferroptosis (Stoyanovsky *et al.*, 2019).

While regulation of iron transport and iron homeostasis is known to affect ferroptosis in animal systems, very little information is available for plant cells so far. In animals, blocking iron acquisition inhibits ferroptosis (Yang and Stockwell, 2008; Gao *et al.*, 2015) whereas knocking down iron efflux transporters is sufficient to accelerate ferroptotic cell death (Ma *et al.*, 2016).

Ferroptosis in plants is also an iron-dependent pathway. Ferroptosis triggered by heat stress can be blocked with iron chelators (Distéfano *et al.*, 2017). Additionally, a recent report showed that exogenous supplementation of iron led to an accelerated ferroptotic cell death in plants infected with the highly virulent tobacco mosaic virus 24A+UPD (Macharia *et al.*, 2020). However, the mechanisms leading to iron accumulation during ferroptotic cell death are still unknown in plants.

Iron transport is quite different in animals and plants (Connorton *et al.*, 2017; Wang *et al.*, 2020). This is not only due to the presence of specific iron transporters involved in iron uptake at the plasma membrane, but also because plant cells contain plastids and a large vacuole that also participate in the regulation of iron homeostasis. In fact, vacuolar iron transporters play essential roles in iron storage and transport (Bashir *et al.*, 2016). In leaves, chloroplasts contain around 70% of total iron, which is mostly associated with thylakoids (Kroh and Pilon, 2020). Since exposing heat-treated seedlings to light accelerates ferroptosis, it was proposed that active chloroplast might have a role in this process (Distéfano *et al.*, 2017). It is also known that expression of chloroplastic iron transporters is induced by light. While chloroplasts display specific iron transporters as PIC1 (permease in chloroplasts 1) (Duy *et al.*, 2011), transporters of the FRO and YSL families are also present (Jeong *et al.*, 2008; Divol *et al.*, 2013). In addition, NEET proteins are proposed to participate in Fe-S/Fe transfer and to play a role in iron homeostasis (Kroh and Pilon, 2020). On the other hand, YSL transporters seem to be involved in iron-nicotinamide release, playing an important role during embryogenesis and senescence (Divol *et al.*, 2013). As in animals, mitochondria shrinkage is a hallmark of ferroptosis in plants (Distéfano *et al.*, 2017). Although mitochondrial ROS production is not always involved, a number of metabolic changes are described in animal mitochondria associated to ferroptosis (Wang *et al.*, 2020). For instance, mitochondrial voltage-dependent anion channels (VDACs), which transport a large variety of compounds such as ions, DNA and tRNA molecules (Hemono *et al.*, 2020), were shown to be direct targets of the ferroptosis inducer erastin (Dixon *et al.*, 2012; Mühlenhoff *et al.*, 2015). Accordingly, not only VDACs were reported to play a role in plant cell death (Homblé *et al.*, 2012), but erastin also induces a ferroptotic type of cell death in plants (Dangol *et al.*, 2019). While the effect of erastin as a ferroptosis inducer was mainly associated with the inhibition of the cystine/glutamate antiporter (system Xc-) in tumor cells (Dixon *et al.*, 2012), the molecular bases underlying the effect of erastin are still elusive in plants. For instance, there are no orthologs for the cystine/glutamate antiporter. However, as explained above, erastin could target plant VDAC channels, which might affect the permeability of the outer mitochondrial membrane as reported in cancer cells (Yang *et al.*, 2020).

FRO and specific mitochondrial iron transporters such as MIT1 and MIT2 are involved in iron acquisition in mitochondria (Heazlewood *et al.*, 2004; Wu *et al.*, 2005; Jain *et al.*, 2019). Additionally, Arabidopsis plants harbor a unique member of the NEET proteins family (also named CISDs) which is involved in sulfur and iron efflux and is localized both in mitochondria and chloroplasts (Nechushtai *et al.*, 2012). *AtNEET* knock-down plants show early senescence and accumulate high levels of iron and ROS (Nechushtai *et al.*, 2012). In animals, NEET proteins negatively regulate ferroptosis (Yuan *et al.*, 2016; Kim *et al.*, 2018). Remarkably, recent data show that a specific NEET protein negatively regulates VDAC in a redox dependent manner (Lipper *et al.*, 2019). These data support a putative role of NEET proteins during ferroptosis, modulating mitochondrial iron influx and ROS homeostasis in plants. Finally, ATM3 (a member of the ATP Binding Cassette family) exports glutathione disulfide from mitochondria to the cytoplasm, where it is required for the assembly of cytosolic Fe-S clusters in Arabidopsis (Schaedler *et al.*, 2014). Regulation of this assembly machinery could be important during plant ferroptosis, as it was shown that suppressing Fe-S clusters biosynthesis induces ferroptosis in other systems (Alvarez *et al.*, 2017).

Another mitochondrial protein with conserved functions in mammals, yeast, bacteria and plants is frataxin (Buchensky *et al.*, 2017; Gomez-Casati *et al.*, 2018). In Arabidopsis, frataxin knockout mutants are embryo lethal (Vazzola *et al.*, 2007) while knockdown mutants display a deficiency in Fe–S cluster assembly (Busi *et al.*, 2006) and abnormal iron accumulation (Martin *et al.*, 2009; Jain *et al.*, 2019). In addition, double mutants impaired in frataxin and Fer4 genes suggested that both proteins contribute to the composition of the leaf ionome (Murgia and Vigani, 2015). As in plants, frataxin knockdown human cells also show iron accumulation and aberrant Fe–S cluster assembly (Du *et al.*, 2020). These cells are also more sensitive to erastin induced cell death while frataxin overexpression results in erastin resistance, although they are still sensitive to RSL3 (Du *et al.*, 2020).

Ferritins are iron-storage proteins that are generally targeted to chloroplasts (Briat *et al.*, 2010), although localization of a specific isoform, Fer4, has been also documented in mitochondria (Tarantino *et al.*, 2010). In Arabidopsis there are four ferritins whose

expression is not only regulated by tissue or developmental stage, but also by environmental conditions, iron availability or wounding (Ramirez *et al.*, 2011). By buffering excess of iron in the cell, ferritins also protect the cells against oxidative stress. This has been extensively shown in different plant species (Ravet *et al.*, 2009; Yadav *et al.*, 2017; Zang *et al.*, 2017). Remarkably, it was recently demonstrated that silencing ferritin encoding genes results in accelerated ferroptosis upon virus infection in tobacco plants (Macharia *et al.*, 2020). In agreement, overexpression of an At-Ferritin enhances tolerance to heat stress in Arabidopsis and wheat by reducing the oxidative stress triggered by high temperatures (Zang *et al.*, 2017). These reports support a role for ferritin not only in iron homeostasis and oxidative stress moderation, but also playing an active role in plant ferroptosis. A similar role has been proposed for ferritins in mammalian cells, where it was shown that genetic inhibition of the ferritin heavy chain 1 (FTH1) promotes erastin-induced ferroptosis (Hao *et al.*, 2018). In addition, ferroptosis-sensitive cells show decreased ferritin expression (Hao *et al.*, 2018). Ferritin degradation was also shown to contribute to ferroptosis, increasing the levels of labile iron in fibroblasts and cancer cells (Gao *et al.*, 2016; Hou *et al.*, 2016).

GSH depletion has been largely recognized as a ferroptosis hallmark both in plants and animals (Distéfano *et al.*, 2017; Stockwell *et al.*, 2017). In mammalian cells, the main effect of such GSH drop is GPX4 inactivation (Yang *et al.*, 2014), which leads to the accumulation of toxic lipid peroxides. However, increasing evidence supports the notion that GSH might have additional new functions related to cellular iron homeostasis. Through the formation of iron complexes, GSH is able to sense and regulate iron levels, iron transport, and the biosynthesis of iron cofactors in mammalians and yeast (Berndt and Lillig, 2017; Patel *et al.*, 2019).

GSH may also play a role in nitric oxide (NO) accumulation, as the synthesis of a major cellular NO reservoir, S- nitrosoglutathione (GSNO), requires glutathione (Shanmugam *et al.*, 2015). In addition, GSH forms a mixed of mono- or di- thiol dinitrosyl- Fe complexes (MNIC and DNIC) which facilitates its diffusion and/or transport from cell to cell (Watts and Richardson, 2001; Buet *et al.*, 2019). These MNIC/DNIC complexes together with Glutathione- associated NO- mediated S- nitrosylation of

proteins were shown to mediate the signaling cascades that are triggered in response to iron deficiency (Ramirez *et al.*, 2011; Shanmugam *et al.*, 2015; Buet *et al.*, 2019). When GSH levels are low, a situation that takes place during ferroptosis, there is a drop in NO and GSNO levels in Arabidopsis (Shanmugam *et al.*, 2015). In addition, it has been shown that the enzymatic degradation of GSNO via GSNOR is required to provide tolerance to iron excess (Li *et al.*, 2019). Although these molecules were not traditionally associated to animal ferroptosis, a recent report showed that inducible nitric oxide synthase (iNOS) modulates susceptibility to ferroptosis in macrophages and microglia (Kapralov *et al.*, 2020). Inactivation of iNOS confers sensitivity, whereas NO donors confer resistance of cells to ferroptosis. The anti-ferroptotic effects of NO in these cells were attributed to its ability to react with lipid radicals generated by 15-LOX (Kapralov *et al.*, 2020).

In addition, increasing evidence supports a role of glutaredoxins (GRXs) regulating iron homeostasis. GRXs are ubiquitous disulfide oxidoreductases that play an important role in the response of plants to oxidative stress, catalyzing the reduction of disulfide bonds and regulating redox status (Rouhier *et al.*, 2004). Specifically, class II GRXs bind Fe-S clusters, taking part in their biogenesis and also in their transfer to Fe-S-containing enzymes (Rouhier *et al.*, 2007; Moseler *et al.*, 2015). Arabidopsis AtGRXs14, 15, 16, and 17 all can bind Fe–S clusters (Iñigo *et al.*, 2016; Ströher *et al.*, 2016). Remarkably, GRXs17 shows redox-dependent holdase activity, releasing Fe-S clusters upon stress (Wu *et al.*, 2017; Martins *et al.*, 2020). Accordingly, class II GRXs might constitute a plausible source of labile iron during ferroptosis, as high temperatures induce the release of 2Fe-2S clusters (Wu *et al.*, 2017; Martins *et al.*, 2020). Also, an imbalance in iron-sulfur cluster assembly could also lead to iron accumulation, priming cells to ferroptosis as observed in tumor cells (Du *et al.*, 2020). Interestingly, silencing *glutaredoxin 5* (*GLRX5*) activated iron-starvation response in mouse models, boosting up intracellular free iron predisposing cells to ferroptosis (Lee *et al.*, 2020).

Iron can also bind to Poly rC Binding-Proteins (PCBPs), which in humans were found to transfer iron to the lipoxygenases implicated in ferroptosis (Stoyanovsky *et al.*, 2019). Supporting a similar role in plants, ferroptosis triggered upon virus infection

involves a strong induction of a PCBP protein in *Nicotiana Benthamiana* (Macharia *et al.*, 2020).

Transcription factors from the bHLH family are also implicated in iron sensing, regulating the expression of iron-related genes, such as iron transporters FRO and IRT (Zhang *et al.*, 2019). These transcription factors are induced in response to iron deficiency (García *et al.*, 2018; Rodríguez-Celma *et al.*, 2019). Interestingly, iron deficiency in combination with heat stress downregulates the expression of iron deficiency-responsive genes, which might ameliorate heat stress-induced ferroptosis in plants (Buckner *et al.*, 2019). Similarly, silencing of *IREB2*, which encodes a protein that senses iron levels and controls the expression of many other proteins related to iron transport, storage and turnover, attenuates erastin-induced ferroptosis in cancer cells (Dixon *et al.*, 2012).

Lastly, iron is also essential to regulate many metabolic reactions, as it is part of key cofactors such as heme groups, Fe-S clusters and non-heme iron centers. Chloroplasts and mitochondria are rich in Fe-S proteins, while peroxisomes and the endoplasmic reticulum contain heme proteins such as peroxidases and cytochrome P450s; and mono- and di-iron enzymes are found in all cell compartments (Connorton *et al.*, 2017). Many of those co-factors are required for enzymatic activities involved in the execution of ferroptosis (Stockwell et al., 2017). Besides the LOXs activity already described, other iron containing enzymes, such as cytochrome P450 oxidoreductases, were also identified to mediate PUFAs peroxidation (Zou *et al.*, 2020). Furthermore, it was observed that suppressing iron-sulfur clusters biosynthesis induces ferroptosis in lung tumors (Alvarez *et al.*, 2017). Interestingly, most of the plant metabolically-active iron is included in these clusters and the selective protein degradation that takes place during abiotic stresses was shown to have an impact on iron metabolism (Wawrzyńska and Sirko, 2020).

Concluding Remarks

Ferroptosis in plants shares all the main hallmarks described for ferroptosis in other systems. As described above, it occurs through GSH depletion, ROS accumulation and iron-dependent lipid peroxidation. Iron plays an essential role in ferroptosis. Accordingly, several proteins involved in iron homeostasis were shown to be modulated during plant ferroptosis or to regulate cell death (Table 1). In addition, growing evidence supports an active role for several iron-storage proteins and iron transporters during plant ferroptosis, as reported for animal systems (Table 1). The particularities of the plant cell also add more complexity to the ferroptosis pathway, as different compartments might contribute not only as an iron source but also as sites of localized ROS accumulation or by providing PUFAs containing membrane lipids (Figure 1). How these lipid peroxides act as pro-ferroptotic signals is a question that still remains to be elucidated. However, recent reports suggest that they might interact with protein targets causing the formation of pores in plasma membranes or even their rupture (Stoyanovsky *et al.*, 2019).

Ferroptosis can be triggered by abiotic or biotic stresses. Recent reports showed the relevance of ferroptosis during plant-pathogen interactions (Dangol *et al.*, 2019; Macharia *et al.*, 2020). Interestingly, the role of iron homeostasis in plant disease has been largely documented in several systems (Greenshields *et al.*, 2007; Douet *et al.*, 2009; Dellagi *et al.*, 2009). As iron-dependent cell death seems essential for restricting pathogen growth and plant infection, iron sequestering from the host might be a successful strategy for infection. Concordantly with this idea, high-affinity iron uptake mechanisms such as siderophore-mediated iron uptake have been reported to be essential for virulence in fungi and bacteria (Greenshields *et al.*, 2007; Dellagi *et al.*, 2009).

Heat stress is also able to induce ferroptosis in a very specific manner (Distéfano *et al.*, 2017). Even when there is not a master regulator described for stress response in plants, several metabolic and transcriptional changes are commonly seen in response to different types of environmental stimuli (Buckner *et al.*, 2019). Ferroptosis, in fact, involves molecular events such as ROS accumulation, activation of lipid peroxidation and antioxidant depletion that have been described after several kinds of insults (Senda and Ogawa, 2004; Pavet *et al.*, 2005; Van Breusegem and Dat, 2006; Doyle *et al.*, 2010; Noctor *et al.*, 2012; Xie *et al.*, 2014). As knowing the kind of stimuli that triggers ferroptosis would provide with new insights for this cell death pathway, revisiting already described regulated cell death processes might contribute to a better understanding of the implications of ferroptosis in plants.

Data Availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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Table 1. Proteins/compounds involved in iron sensing, transport and homeostasis that might have an active role modulating plant ferroptosis.

Iron-related activity	Protein/ compounds	Subcellular compartment	References
Iron transporters	VDAC	Mitochondrion	(Homblé *et al.*, 2012) (Dangol *et al.*, 2019)
	NEET	Mitochondrion	(Nechushtai *et al.*, 2012)
Iron storage proteins/Iron Scavengers	Ferritin	Plastids	(Macharia *et al.*, 2020) (Zang *et al.*, 2017)
Iron chaperons	Frataxin	Mitochondrion	(Busi *et al.*, 2006) (Martins *et al.*, 2020)
	GRXs Class II	Plastid/Mitochondrion/Cytosol	
	PCBP	Cytosol	(Macharia *et al.*, 2020)
Iron containing enzymes	LOX	Cytosol	(García-Marcos et al., 2013a)
Iron levels modulators	BURTUS	Nucleus	(Buckner *et al.*, 2019)
	POPEYE	Nucleus	(Buckner et al., 2019)
	GSH	Cytosol	(Distéfano *et al.*, 2017)
	GSH-NO-Fe	Cytosol	(Buet *et al.*, 2019)
	GSNOR	Cytosol	(Li *et al.*, 2019)

Figure Legends

Fig. 1. Proposed model for ferroptosis in plants. Ferroptosis is induced upon heat and biotic stress. The pathway involves GSH depletion, ROS accumulation and iron-dependent lipid peroxidation. Cytoplasmic retraction, normal nuclei, mitochondria shrinkage, and the formation of small lytic vacuoles are the main morphological hallmarks of plant ferroptosis. Iron accumulation might implicate different mechanisms and subcellular compartments. Several proteins related to iron homeostasis are modulated during plant ferroptosis, such as ferritins and PCBPs proteins. Voltage-dependent anion channels (VDAC) and NEET proteins, ATM3 transporters, frataxin and the iron-sensitive proteins POPEYE (PYE) and BRUTUS (BTS) might also have a significant role. An oxidative burst occurs via cytoplasmic ROS accumulation through NADPH oxidase activity (NOX) and the generation of toxic lipid- peroxides. Those lipid peroxides may result from enzymatic or non- enzymatic processes on membrane poly-unsaturated fatty acids (PUFAs), involving either lipoxygenases (LOXs) activity or Fenton chemistry. However, where lipid peroxidation occurs or which specific lipids undergo peroxidation, it is still unknown. Glutathione peroxidase (GPX) detoxifies lipid peroxides, acting as a ferroptosis negative regulator. GPX uses thioredoxin (TRX) as a main reductant agent, but it might also utilize GSH. GSH is also required for nitric oxide (NO) accumulation, since the synthesis of a major cellular NO reservoir, GSNO, requires GSH. Lipid peroxides act as pro-ferroptotic compounds, although their targets are still unknown. However, recent evidence suggests that they might interact with membrane proteins forming pores and damaging the plasma membrane. CPX, Fer-1, Lip-1 and DPI inhibit ferroptosis. Erastin and RSL3 are ferroptosis inducers. Dashed lines indicate indirect evidence.

Figure 1