Distéfano et al., https://doi.org/10.1083/jcb.201605110

THE JOURNAL OF CELL BIOLOGY

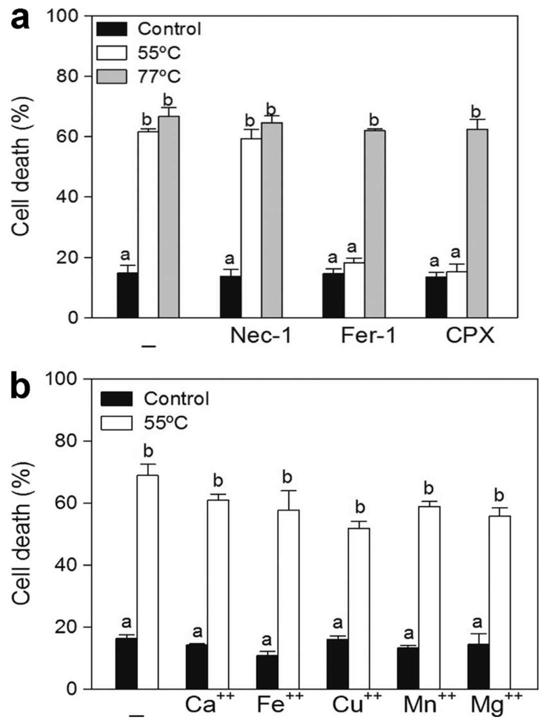


Figure S1. **Effect of the necroptosis inhibitor Nec-1 and divalent transition metal ions on cell death induced by a 55°C HS treatment.** (a) Effect of Nec-1 on cell death triggered by 55°C or a 77°C HS treatment. 6-d-old seedlings were preincubated overnight (16 h) with 20 µM Nec-1, 1 µM Fer-1, or 10 µM CPX. (b) 6-d-old seedlings were preincubated overnight (16 h) with different divalent transition metal ions as indicated. (a and b) Cell death was induced by treatment at 55°C for 10 min. Root hairs were stained with Sytox green, and dead root hairs were quantified. Results are expressed as a percentage of dead cells. Data are the mean ± SEM of three independent experiments. Different letters denote statistical difference (one-way analysis of variance, $P < 0.05$).

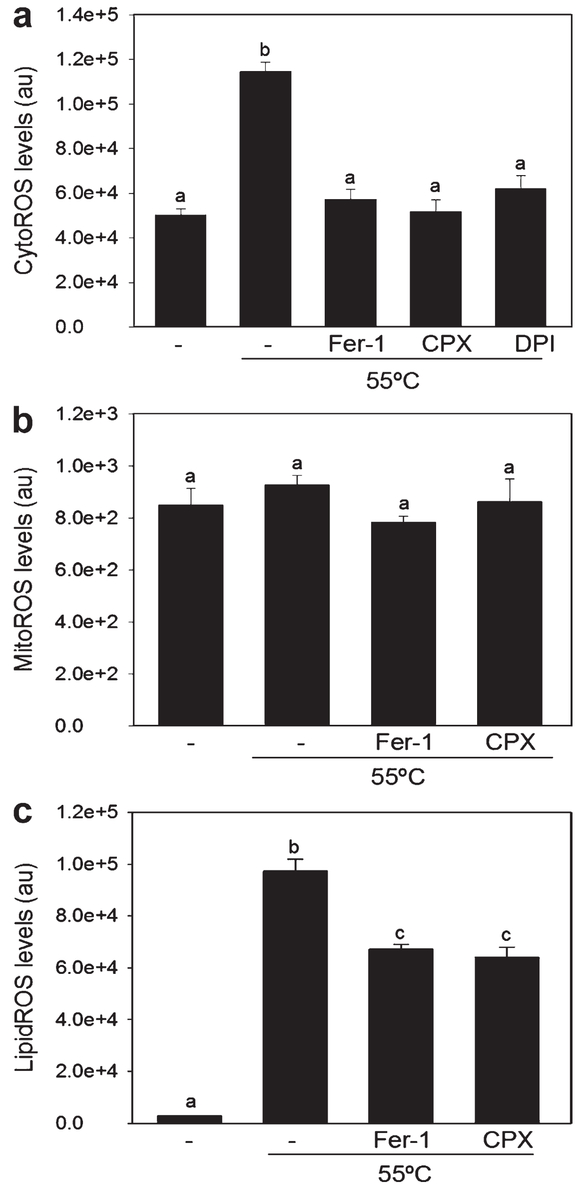


Figure S2. **55°C HS in *Arabidopsis* cell suspensions triggers the accumulation of ROS, which can be inhibited by Fer-1, CPX, and DPI.** 7-d-old cultures were pretreated overnight (16 h) with 1 µM Fer-1, 10 µM CPX, or 10 µM DPI as indicated and treated at the specified temperature. (a) Cytosolic ROS (CytoROS) levels were detected with the H2DCFDA probe 3 h after a 55°C treatment. (b) Mitochondrial ROS (MitoROS) levels were detected with the mitoSOX probe 3 h after a 55°C treatment. (a and b) Data are the mean ± SEM of three independent experiments. Different letters denote statistical difference (one-way analysis of variance, $P < 0.05$). (c) Lipid ROS levels were detected with the C11-BODIPY probe 3 h after 55°C treatment. Data are the mean ± SEM of three independent experiments. No significant differences were found (one-way analysis of variance).

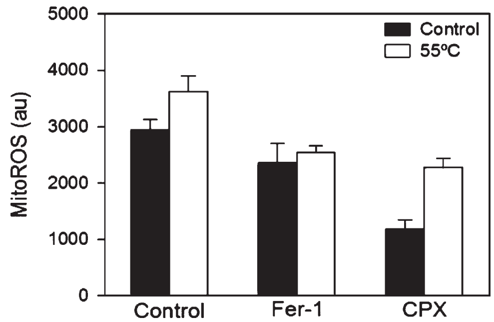


Figure S3. **MitoSOX is not involved in the cell death triggered by 55°C treatment in *Arabidopsis* root hairs.** Mitochondrial ROS (MitoROS) levels were detected with the mitoSOX probe 3 h after 55°C treatment. Data are the mean ± SEM of three independent experiments.

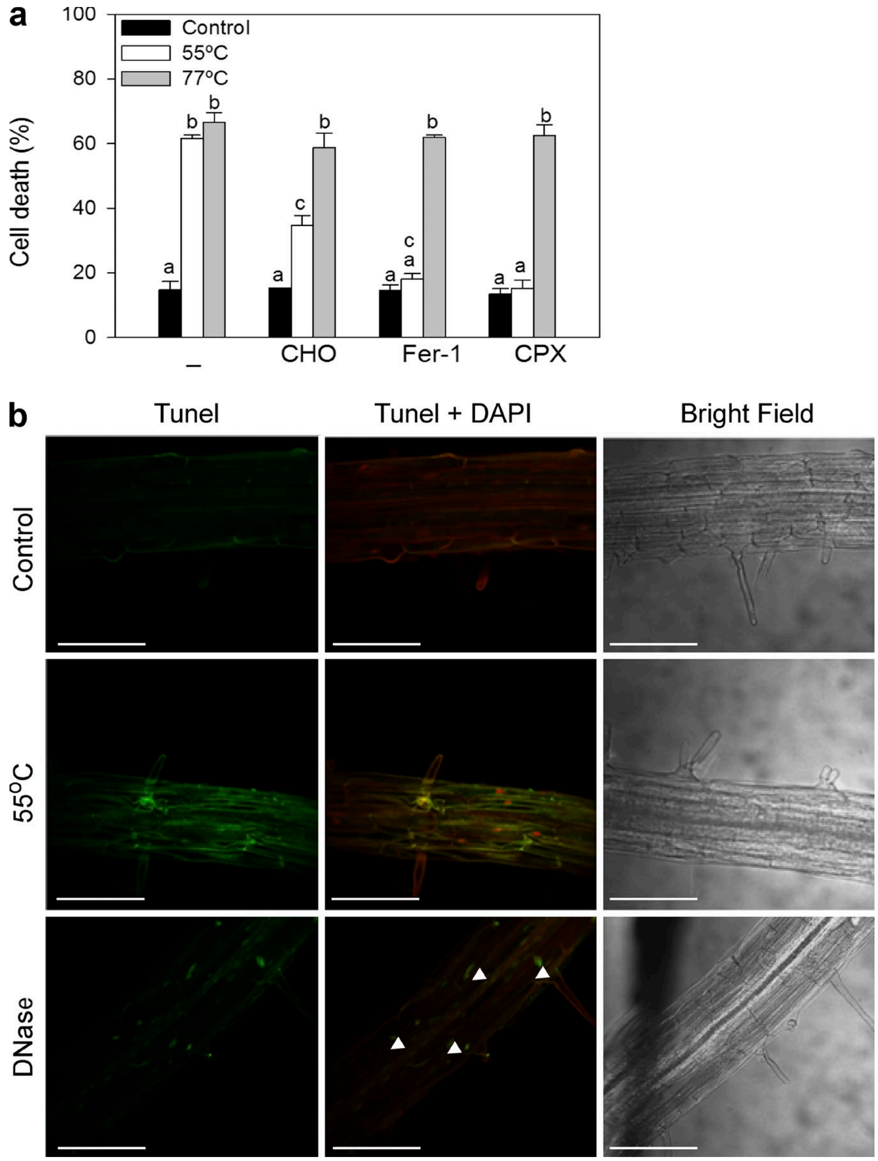


Figure S4. **A caspase-like activity is required for cell death after a 55°C HS, but DNA fragmentation is not detected.** (a) Effect of the caspase-3 inhibitor CHO on rates of HS-induced cell death in *Arabidopsis* root hairs. 6-d-old seedlings were preincubated in a 1 µM CHO solution or with 1 µM Fer-1 or 10 µM CPX for 16 h before HS at 55°C or 77°C. Root hairs were stained with Sytox green, and dead root hairs were quantified. Results are expressed as a percentage of dead cells. Data are the mean ± SEM of three independent experiments. Different letters denote statistical difference (one-way analysis of variance, $P < 0.05$). (b) TUNEL labeling of *Arabidopsis* roots showed no TUNEL-positive nuclei in roots submitted to 55°C HS. Arrowheads indicate positive TUNEL nuclei. Bars, 100 µm.

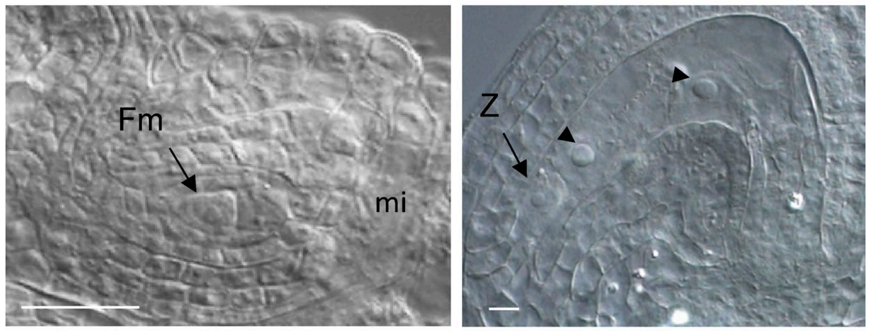
a

Treatment	Remaining spores	Only FM
Mock	4	118
CPX	6	119
Fer-1	4	108

b

Treatment	Fertilized ovules	Unfertilized ovules
Mock	64	17
CPX	59	12
Fer-1	62	21

c



d

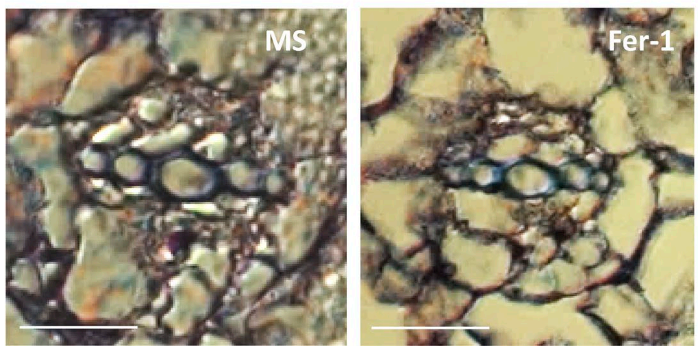
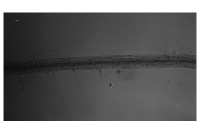
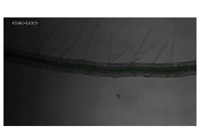


Figure S5. **Effect of ferroptosis inhibitors on reproductive and vascular development in *A. thaliana.*** (a) Megaspore cell death was analyzed in pistils from *Arabidopsis* inflorescences that were treated with either 10 µM CPX or 1 µM Fer-1 (in 0.1% DMSO and 0.01% Silwet L-77) or a mock solution (0.1% DMSO and 0.01% Silwet L-77). (b) Fertilization as a sign of normal synergid cell death was analyzed in pistils from *Arabidopsis* inflorescences that were treated with either 10 µM CPX or 1 µM Fer-1 (in 0.1% DMSO and 0.01% Silwet L-77) or a mock solution (0.1% DMSO and 0.01% Silwet L-77) that showed signs of pollination (pollen on the stigma) 3 d after treatment. (c) DIC images of a developing ovule showing a functional megaspore (Fm; left) and a fertilized embryo sac showing a zygote (Z) and endosperm nuclei (arrowheads). Mi, micropyle. Bars, 25 µm. (d) The effect of ferroptosis inhibitors on xylem anatomy was analyzed in hypocotyls of 6-d-old plants that were grown in Murashige and Skoog medium or alone or with 1 µM Fer-1. 50 plants were used for observations in each case. No differences were observed. Bars, 50 µm.

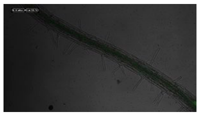


Video 1. **Sytox green staining of the different cell types that compose the root right after a 55°C HS treatment (T0).** The video is derived from a stack of serial confocal z sections taken through the root of a 6-d-old seedling stained with SYTOX green after a 55°C HS (4-µm step size). This video supplements Fig. 2 b.

Video 2. **Sytox green staining of the different cell types that compose the root 2 h after a 55°C HS treatment.** The video is derived from a stack of serial confocal z sections taken through the root of a 6-d-old seedling stained with SYTOX green 2 h after a 55°C HS (4-µm step size). This video supplements Fig. 2 b.



Video 3. **Sytox green staining of the different cell types that compose the root 4 h after a 55°C HS treatment.** The video is derived from a stack of serial confocal z sections taken through the root of a 6-d-old seedling stained with SYTOX green 4 h after a 55°C HS (4-µm step size). This video supplements Fig. 2 b.



Video 4. **Sytox green staining of the different cell types that compose the root 6 h after a 55°C HS treatment.** The video is derived from a stack of serial confocal z sections taken through the root of a 6-d-old seedling stained with SYTOX green 6 h after a 55°C HS (4-µm step size). This video supplements Fig. 2 b.

Table S1. **Identity of the transcriptional pharmacodynamic ferroptosis markers reported in Dixon et al. (2014) and putative orthologues found in *A. thaliana***

Ferroptosis marker	Description	*Arabidopsis* blast hits (e value)
ChAC1	Mammalian proapoptotic protein of unknown function induced during endoplasmic reticulum stress	AT4G31290 (7e-30), AT1G44790 (2e-36), AT5G26220 (8e-28)
DDit4	DNA damage–inducible transcript 4	
LOC284561	Uncategorized gene affiliated with the lncRNA class	
Asparagine synthetase	Encodes asparagine synthetase (EC 6.3.5.4)	AT5G65010 (2e-115), AT3G47340 (2e-114), AT5G10240 (2e-114)
TSC22D3	Encodes for a leucine zipper protein, functions as transcriptional regulator	
DDIT3	DNA damage–inducible transcript 3	
JDP2	HUMAN isoform 2 of Jun dimerization protein 2	
SESN2 gene	Member of the sestrin family of PA26-related proteins	
SLC1A4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	
txnip	Encodes for a thioredoxin-interacting protein	
VLDLR gene	Encodes a lipoprotein receptor that is a member of the LDLR family	
GPT2 gene	Alanine aminotransferase: catalyzes the reversible transamination between alanine and 2-oxoglutarate to form pyruvate	AT1G72330.1, mitochondrion, 6e-150; AT1G23310.1 apoplast, chloroplast, cytoplasm, membrane, peroxisome, vacuole, 3e-147; AT1G70580.4 chloroplast, chloroplast, stroma, cytoplasm, peroxisome, 3e-145; AT1G17290.1 chloroplast, mitochondrion, 2e-142

Table S2. **List of primer pairs used for quantitative PCR**

Gene	Forward, 5′ to 3′	Reverse, 5′ to 3′
ASN1	GGGATGCAAGCTGGTCCAACA	TGACCCATCATCATCGGCATGT
ASN2	GCTGTAGAATGGGATGCAACTTGGT	TCCTCAATGCCTGTAGTGTTGTTCT
ASN3	ACGCAGCTTGGTCACAGAATCT	CCTCAAACAATGGCTGGAGTCTTCT
CCL1	TCAGGGCCAAACAGAGACTA	CGGTGGCATCAATGTCTAAC
CCL2	GGGCATGAGGAGGACTATGT	GGCTTGCTCTTGATTCCTTC
CCL3	CCAGCTCCATTGGAAGAAAT	TACTCTCGGTTGTTCCCACA
GPT2a	AGTTGTAGTCCCTGGTTCTGGCT	AGCTCTTGTGGAACTCTGTCAGACG
GPT2b	TCCCTTGCCTTCACCTTCCACA	AGCCAGAACCAGGGACAACGA
VDAC1	GAACGACAAGGGCGATCTAT	TAACTTGTGGCTCACTTCGG
VDAC2	GGCCCTTACGTCTACTGCTC	ACTTGTATTGGGTGGCAACA
VDAC3	GGCCCTGAAGTGGGTAGA	AAGATTGTTTAGCAAACCAGACA
VDAC4	ATCACAAGTCTGGCAAGCTG	ACAGAGGAGTTGGGTTGAGG
VDAC5	GGGTATGACACCACATCTCG	GGCTTTGATCGAATCTCCTT
KOD	TATGTGGTGGCTAGTTGGACTTACA	AGCTTTACTTAGAGATGACAGAGACGCT
BAX-1	CCCTTGATCAAAGTGGCAAT	GAAAGCAGTCCTCCAAGGTAGAG
HRD1	GCTCAAGGAAGGTCAATGGG	CAAGGGAAGGCCATAGTTCA
SEL1	GGAATATGCAGTCGAAGATGG	CACCGGAGAATCCTTTGAGA
PR2	GCAATGCAGAACATCGAGAA	TCATCCCTGAACCTTCCTTG
PR5	GCCGTGGAGCTAACGATAAG	GCGTAGCTATAGGCGTCAGG
WRKY33	TGGAGAGAGCATCACACGAC	GTGCTCTGTTTGTGGCGTAA
LSD1	TGTCAAACTACGAACCTTGTGC	TTGATCTGCGCAACCTGA
ACS2	GCTGGTTTATTTGCGTGGAT	AGGAAGAGCCAGGAGACACA
ATG7	TGTTACACGCCCGGGTCTAG	GAGTTCAACGGCGAGAGCTC
ATG8	ATTGTCGAAAGAGCCGAGAA	GATCCGCTTCCGTACAACAT
ATG9	AGACCCTTGAGTGGACCCTT	AGCCCAACCACAAATAGTCG
VPE	AAAGGTGGAAGCGGTAAGGT	TATGTGAGGCGTATTTGGCA
MC1	CCGAGGAAGAAACTGATCCA	GTTTCTTTGACGCGAACCAT
MC2	CGTGGACGATGAGATCAATG	TCCGAGCCTGTCCATTCTAC
RHD6	CCTAAATCCGCTGGAAACAA	TGTTGGCTTAGGCTTGGTCT
WER	GCTCCACAAGTTGCTTGGTA	TCACCATTGCTCTGTTTGGT
Bip1	TGCCTTTGAGCATCATTGAA	TCAGTCCTGAGGAGATTAGTGCT
Bip2	TCTCCTCAGGACTGAAAACCT	TCGACGTTAAGAGATTGATCG
Bip3	GGCTTCCCATCTTTGTTCAC	CGAAACGTCTGATTGGAAGAA
Bzip60	TCTCAAGCATTCTCTTTCGAGAT	CGATGATGCTGTGGCTAAAA