Autophagy is essential for optimal Fe translocation to seeds in *Arabidopsis*

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Author contributions

S.T., C.M.D., and M.P. designed the experiments. M.P. performed most of the experiments. J.D. developed the analysis of the $^{56}Fe/^{57}Fe$ ratios and performed isotopic measurements. M.P. and S.T. analyzed the data. M.P. wrote the paper with contributions of all the authors. S.T. supervised and complemented the writing.

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Abstract

Micronutrient deficiencies affect a large part of the world population. They are mostly due to the consumption of grains with insufficient content of Fe or Zn. It is therefore important to improve our knowledge of the mechanisms of micronutrient loading to seeds. Nutrient loaded in seeds originate either from *de novo* uptake by roots or recycling from leaves. Autophagy is a conserved mechanism for nutrient recycling in eukaryotes and was shown to be involved in nitrogen remobilization to seeds. Measuring the distribution of metal nutrients at the end of the life in *Arabidopsis thaliana* plants impaired in autophagy, we have investigated the role of autophagy in metal micronutrient translocation to seeds. We found that several Arabidopsis genotypes impaired in autophagy display defects in nutrient remobilization to seeds. In *atg5-1*, which is completely defective in autophagy, the efficiency of Fe translocation from vegetative organs to seeds was severely decreased even when Fe was provided during seed formation. Combining *atg5-1* with *sid2* mutation that counteracts premature senescence associated to autophagy deficiency and using ^{57}Fe pulse labelling, we could propose a two-step mechanism in which iron taken up de novo during seed formation is first accumulated in vegetative organs and subsequently remobilized to seeds. Finally, we showed that translocations of zinc and manganese to seeds are also dependent on autophagy. Our results highlight the importance of autophagy for optimal micronutrient remobilization to seeds. Fine tuning autophagy during seed formation opens new possibilities to improve this trait.

Introduction

Metal micronutrients are essential to all forms of life. For instance, iron (Fe) plays a major role in oxido-reduction reactions allowing respiration in mitochondria and photosynthesis in chloroplasts (Nouet et al., 2011). Worldwide, about 2 billion people suffer from Fe deficiency which affects mostly children and women in developing countries (WHO, 2016). Fe deficiency is most often due to the major place taken by staple food crops in human diet, although they are poor sources of this micronutrient (Murgia et al., 2012). During the last decade, different biofortification strategies such as fertilization, conventional breeding, and genetic engineering, were investigated in an attempt to increase key micronutrient levels in seeds of crop species (Murgia et al., 2012).

The improvement of micronutrient loading in seeds requires knowledge of micronutrient trafficking pathways within the plant, including uptake from soil and remobilization from senescing organs. Most studies have so far focused on micronutrient uptake in root cells, intracellular partitioning, and long-distance transport. In contrast, micronutrient remobilization from vegetative organs during senescence, has received much less attention (Pottier *et al.*, 2014). Senescence of *Arabidopsis thaliana* is associated to decreases by 50% of leaf Fe concentration, in parallel with micronutrient filling in seeds (Himelblau and Amasino, 2001). This finding implies that 50% of micronutrients present in senescent leaves are not remobilized. A better understanding of micronutrient remobilization from senescent organs is therefore likely to highlight new solutions to improve seed micronutrient content. More specifically, mechanisms controlling the availability of nutrients in source organs such as autophagy, could be a matter of great significance for micronutrient loading in seeds (Shi et al., 2012; Pottier et al., 2014) .

Autophagy is conserved from yeast to animals and plants. It allows degradation of cytoplasmic components by lysosomal (animals) or vacuolar (plants) internalization mediated by double membrane vesicles called autophagosomes (Reumann et al., 2010). Autophagy promotes energy production, toxic components elimination, and nutrient recycling by discarding aberrant proteins, damaged organelles as well as normal cytoplasmic components when they are no longer useful (Yoshimoto, 2012). Autophagy related genes (*ATG*) involved in autophagosome formation were first identified in yeast (Matsuura *et al.*, 1997). Subsequently, orthologues of *ATG* genes were identified in plants and autophagy deficient plants were characterized (Li and Vierstra, 2012). All autophagy deficient plants exhibit

hypersensitivity to carbon and nitrogen starvation pointing to a central role of autophagy for nutrient recycling (Liu and Bassham, 2012; Guiboileau et al., 2013). The up-regulation of *ATG* genes during leaf senescence in *Arabidopsis* suggests a role for autophagy in nutrient recycling during leaf senescence (Doelling et al., 2002; van der Graaff et al., 2006; Chung et al., 2010; Breeze et al., 2011; Liu and Bassham, 2012). In agreement with this hypothesis, Nitrogen Remobilization Efficiency, i.e. the proportion of nitrogen allocated to seeds through remobilization from vegetative tissues during senescence, is strongly affected in autophagy deficient plants (Guiboileau et al., 2012).

In this work, we investigated the role of autophagy in metal micronutrient resorption from senescent leaves to seeds in *Arabidopsis*. Our results indicate that micronutrient remobilization is impaired in vegetative tissues of autophagy deficient plants. Focusing on ATG5, which is essential for autophagosome formation (Kirisako et al., 2000), we show that autophagy-dependent remobilization is critical for optimal translocation of Fe as well as other metal nutrients from vegetative organs to seeds.

Results

Autophagy deficient plants retain higher concentrations of zinc, manganese, and iron concentrations in vegetative organs

To investigate the involvement of autophagy in micronutrient remobilization from vegetative organs to seeds during senescence, we first compared metal concentrations in vegetative organs of wild-type and several autophagy deficient plants after the completion of the plant's life cycle (Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005; Guiboileau et al., 2012). If remobilization of metal micronutrients to seeds is impaired in plants with compromised autophagy, their concentration in the dry remains of vegetative parts should be increased compared to wild-type. Manganese (Mn), iron (Fe), and zinc (Zn) concentrations were up to 2.5 times higher in dry remains of autophagy deficient plants compared to those of wild-type plants (**Fig. 1**). Accordingly, an increased level of FERRITINS (**Supplemental Fig. S1**), which are involved in Fe storage (Briat et al., 2010), was observed in vegetative tissues of *atg5-1* mutant compared to those of wild-type plant. The highest increases in metal concentrations were consistently observed in *atg5-1* and *atg4* mutants, which are fully defective in autophagosome formation (Yoshimoto et al., 2004; Guiboileau et al., 2012). In contrast, in dry remains of *atg9.1*, *atg9.2*, and ATG18 RNAi, increases in Mn, Fe, and Zn concentrations were smaller and, in some cases, not significant.

This is consistent with the observation that autophagic bodies have been detected in *atg9.1*, *atg9.2*, and ATG18 RNAi plants, indicating that autophagy is not fully compromised in these mutants (Yoshimoto et al., 2004; Guiboileau et al., 2012).

These data suggest that defects in autophagy affect micronutrient metal resorption from vegetative organs. The severity of the resorption defects appears to be related with the level of autophagy deficiency in the different genotypes tested.

Distinct mechanisms lead to decrease in Fe concentration in seeds of autophagy deficient plant depending on Fe supply during the reproductive stage

To further analyze the role of autophagy in micronutrient resorption from vegetative organs to seeds, we chose to focus on *atg5-1* mutant, which exhibited robust increases in metal concentrations in its dry remains (**Fig. 1**), and on Fe, a critical micronutrient that is often not sufficiently available in seeds of crop plants for human nutrition (Velu et al., 2014). Because autophagy operates a negative feedback loop modulating salicylic acid (SA) signaling to negatively regulate senescence, autophagy deficient plants exhibit an premature senescence phenotype which may interfere with nutrient resorption (Yoshimoto et al., 2009; Guiboileau et al., 2012). Hence, we also included in our analyses the stay-green mutant *sid2*, which is defective in SA biosynthesis, and *atg5sid2* double mutant, in which autophagy is defective while senescence is delayed (Wildemuth et al., 2001; Yoshimoto et al., 2009).

Seed filling may depend on both remobilization from senescent organs and *de novo* uptake by roots during seed formation. In order to discriminate the two pathways, plants were grown in parallel under two different conditions: some were supplied with sufficient Fe during all their life cycle (Fe sufficient supply), while other were deprived of Fe from the onset of flowering on (Fe deprivation).

In order to determine the contribution of autophagy to Fe remobilization from different organs and subsequent Fe loading in seeds, Fe concentrations were measured in leaves, stems, and seeds of *atg5-1*, *atg5sid2*, *sid2* mutants, and wild-type plants. No differences in Fe concentration were observed between *sid2* and wild-type plants, irrespective of the Fe nutrition regime. However, under Fe sufficient nutrition, Fe concentration in *atg5-1* mutant was significantly higher than that of wild-type in leaves (1.8 fold) and in stems (3.4 fold) (**Fig. 2**), in agreement with the results presented in **Figure 1**. The increase in Fe concentration in *atg5-1* vegetative organs was associated with a decrease in seed Fe concentration (**Fig. 2**). Similar results were observed in an independent experiment comparing wild-type and *atg5-1* mutant plants performed under different lighting conditions (**Supplemental Fig. S2**), confirming the

robustness of this observation. Both the increase in Fe concentration in vegetative organs and the decrease in seeds observed in *atg5-1* were reversed to a large extent in *atg5sid2*, suggesting that these effects are in part due to early senescence.

Under Fe deprivation, Fe concentrations were lower in leaves and seeds irrespective of the genotype, as expected (**Fig. 2**). In contrast with the Fe sufficient nutrition, Fe concentrations in vegetative organs of *atg5-1* mutant were only moderately increased (1.2 fold) with respect to wild-type, while seed Fe concentration was significantly decreased by 20% in seeds (**Fig. 2**). These effects were not reversed in *atg5sid2* mutant, suggesting that they are due to impaired autophagy rather than early senescence.

Taken together, these data indicate that *atg5-1* mutant is affected in Fe resorption from vegetative organs to seeds by distinct mechanisms under sufficient Fe supply or Fe deprivation during the reproductive period.

Defective autophagy leads to strong decrease in the pool of Fe allocated to seeds

To evaluate the impact of autophagy on redistribution of Fe pools between vegetative organs and seeds, we calculated Fe mass distribution among organs. For this purpose, we first determined the total biomass distribution as well as the harvest index, which is defined as the ratio of seed biomass relative to the whole aerial biomass of the plant after completion of the life cycle. As previously reported (Guiboileau et al., 2012), loss of ATG5 function led to a strong reduction of total dry biomass (2.8 fold; **Supplemental Fig. S3**) and an even more severe defect in seed production causing a decrease in the harvest index (**Supplemental Fig. S4**). These effects were not significantly reversed in *atg5sid2* mutant, and were also observed when plants were deprived of Fe during the reproductive phase (**Supplemental Fig. S3 and S4**).

Taking biomass and Fe concentrations into account, Fe content was determined for each organ (**Fig. 3A**). First, we observed a lower Fe content under Fe deprivation in all genotypes, indicating that *de novo* Fe uptake during seed formation contributes to total Fe content independently of autophagy. (**Fig. 3A**). Although *atg5-1* mutant displayed significantly higher Fe concentrations than wild-type in vegetative organs (**Fig. 2**), its total Fe content was strongly decreased compared to wild-type due to its much lower biomass (**Fig. 3A; Supplemental Fig. S3**). Disrupting *sid2* gene did not restore Fe accumulation in *atg5-1*, indicating that this defect is not due to the premature senescence of *atg5-1* mutant.

Compared to that of wild-type, lower Fe contents were observed in vegetative organs of *atg5-1* (-35%) and *atg5sid2* (-24%) mutants specifically under Fe deprivation but not under Fe sufficient nutrition (**Fig. 3A**). This result indicates that Fe supply during the reproductive stage

provides vegetative organs of autophagy deficient plants with Fe even though they undergo premature leaf senescence. This suggests that Fe is accumulated in vegetative tissues during the early steps of the reproductive stage.

Iron content of seeds from plants growing with sufficient Fe supply was dramatically decreased by -80% and -60% in *atg5-1* and *atg5sid2* mutants compared to that of wild-type, respectively (**Fig. 3A**). Iron content is therefore much more severely affected by the lack of autophagy in seeds than in other organs. While no effect of Fe supply during the reproductive stage was observed in seeds of *atg5-1* plants, this treatment restored Fe content of *atg5sid2* seeds. This result indicates that *de novo* Fe uptake during seed formation can provide seeds with Fe in autophagy deficient plants, but only when premature senescence is prevented. This suggests that such contribution takes place during the late steps of the reproductive stage, when *atg5-1* but not *atg5sid2* displays leaf senescence symptoms.

Impaired autophagy affects translocation of Fe remobilized from vegetative organs to seeds

We then analyzed the impact of the absence of autophagy on the efficiency of total Fe translocation to seeds ($Fe_{seed}/Fe_{total\ plant}$), under Fe sufficient nutrition and Fe deprivation (**Fig. 3B**). The efficiency of total Fe translocation to seeds was indistinguishable between wild-type or *sid2* mutant plants representing 50-55% of the total plant Fe, irrespective of the Fe nutrition regime. In contrast, the efficiency of total Fe translocation was strongly decreased in *atg5* and *atg5sid2* mutants. Under Fe sufficient nutrition, Fe translocation efficiency was decreased by 63% in *atg5-1* mutant and only 35% in *atg5sid2* mutant confirming that premature senescence limits Fe translocation to seeds under these conditions. Under Fe deprivation, total Fe translocation efficiency was decreased to a similar extent (42-48%) in *atg5-1* and *atg5sid2* mutants, indicating conversely that premature senescence has no impact on total Fe translocation when Fe is not provided during the reproductive phase.

In the independent experiment performed under different lighting conditions, similar total Fe translocation efficiencies of 50-55% were measured in wild-type (**Supplemental Fig. S5**). However, in *atg5-1* mutant, the total Fe translocation efficiency was further decreased under Fe deprivation. We hypothesize that the difference in the light conditions may have modified the timing of leaf senescence or amplified the effect of Fe deficiency.

Using Fe deprivation to estimate the relative contribution of root uptake and remobilization from leaves may be biased by the activation of compensatory mechanisms under Fe deficiency (Pottier et al., 2014). Then, to monitor fluxes specifically between senescing

organs and seeds independently of uptake from soil, we performed a ^{57}Fe labelling pulse-chase experiment. A pulse of ^{57}Fe was provided in the nutritive medium of young rosette plants. Pots and substrate were then thoroughly washed to avoid further ^{57}Fe uptake during the chase period. Plants were subsequently watered with nutritive solution containing non-labelled Fe until the end of the life cycle (Fe sufficient nutrition). At the end of the life cycle, the relative abundance of ^{57}Fe was determined in each organ by ICP-MS. Combining $^{57}Fe/^{56}Fe$ ratios with Fe concentrations and dry weights, the efficiency of ^{57}Fe translocation to seeds was calculated ($^{57}Fe_{seed}/^{57}Fe_{total\ plant}$). This value, corresponding to the efficiency of Fe remobilization from vegetative organs to seeds (**Fig. 3C)**, was very similar to translocation efficiency of total Fe (**Fig. 3B)** irrespective of the genotype. This result indicates that, even though Fe is still taken up during the reproductive stage, Fe loading in seeds is fully achieved by remobilization from vegetative organs under our experimental conditions.

The lack of autophagy also impacts Zn and Mn translocation to seeds

Translocation efficiencies of other nutrients were also calculated in plants growing under Fe sufficient nutrition, following the same approach as for Fe. In wild-type plants, Zn, and Mn translocation efficiencies were 38% and 24% respectively (**Fig. 4)**. In *atg5-1* mutant plant, total translocation efficiencies of these elements were both strongly affected with respect to wild-type. Similar results were observed in the independent experiment performed under different light conditions (**Supplemental Fig. S6**). Besides, no significant differences were observed between for *atg5-1* and *atg5sid2* mutant plants (**Fig. 4)**. Thus, a defect in autophagy affects not only Fe translocation but also at various extents translocation of other micronutrients, even in absence of premature senescence.

Discussion

In the present work, we investigated the involvement of autophagy in metal micronutrient remobilization from vegetative organs to seeds. Analyzing a range of autophagy mutants, we found that the defect in nutrient remobilization correlated with the severity of the defect in autophagosome formation (**Fig. 1**). Accordingly, the severe defect in autophagy in *atg5-1* mutant leads to a decrease in seed Fe concentration and a drastic reduction in Fe translocation efficiency to seeds (**Figs. 2 and 3**). Interestingly, this defect could be alleviated by providing Fe to the roots only when *atg5-1* premature senescence was prevented (**Fig. 3A**). This result, together with the outcome of the ^{57}Fe pulse labelling experiment, suggests that the

Fe taken up by the root during seed formation transited to the leaves before being remobilized to the seeds (**Fig. 3B**). Finally, we found that autophagy plays role not only in Fe but also in Zn and Mn translocation to seeds, supporting that the importance of this mechanism for optimal loading of mineral nutrient to seeds (**Fig. 3C**).

Mineral translocation efficiency to seeds are constants in Arabidopsis

Iron translocation efficiency of *Arabidopsis thaliana* Colombia-0 wild-type was within the 50-55% range irrespective of light conditions or Fe nutrition regime (**Fig. 3B; Supplemental Fig. S5**). Fe uptake observed during reproductive stage under Fe sufficient nutrition has therefore no impact on Fe translocation efficiency of wild-type plants (**Fig. 3A and B)**. A previous study also found that half of the Fe content of *Arabidopsis* leaves was remobilized during senescence (Himelblau and Amasino, 2001), even though the ecotype, the photoperiod and the growth conditions were different. Thus, Fe translocation efficiency appears to be remarkably stable in *Arabidopsis thaliana*. All other micronutrients studied in this work exhibited lower total translocation efficiencies than Fe (**Fig. 4; Supplemental Fig. S5**). We however found that Zn is better translocated than Mn, which is also in agreement with results obtained by Himelblau and Amasino (2001). In contrast, Maillard et al. found large variation in leaf mineral nutrient resorption between different species (Maillard et al., 2015). Pottier et al. also found variation in metal resorption from leaves from different poplar genotypes (Pottier et al., 2015). However, remobilization from leaves rather than translocation to seeds was analyzed in these studies.

Vegetative organs are an obligatory stop over for Fe on its way to seeds

The Fe that is taken up *de novo* during the reproductive stage may be loaded first in young parts of the vegetative organs or directly into seeds. The *atg5-1* mutant, which undergoes premature senescence, displayed different total Fe content under sufficient Fe supply and Fe deprivation conditions (**Fig. 3A**). This observation indicates that Fe uptake after the onset of flowering occurs essentially during the early period of the reproductive stage, prior to the senescence of *atg5-1* mutant leaves. Moreover, *atg5-1* mutant accumulated *de novo* absorbed Fe preferentially in vegetative organs while it was translocated more efficiently to seeds when premature senescence is prevented (**Fig. 3A**). These results suggest a two-stage process in which Fe taken up by roots is transiently accumulated in vegetative organs prior to its remobilization to the seeds as illustrated in **Figure 5**. This hypothesis also accounts for the similar values observed for total Fe and ^{57}Fe remobilization efficiencies in the different genotypes after pulse labelling during the vegetative phase. Waters and Grusak (2008) concluded that *de novo* uptake is at least as important for seed filling as remobilization.

However, they were using different conditions and did not consider the possibility that minerals have to transit to vegetative organs before being translocated to seeds.

Mastering autophagy to improve seed nutrient content

We found that autophagy is a crucial component of micronutrient filling to seeds and thus a strong determinant of seed quality. Although autophagy was shown to be induced during senescence (van der Graaff et al., 2006; Breeze et al., 2011; Htwe et al., 2011), significant amounts of nutrients present in vegetative organs, 30% of N in optimal conditions, 46% of Fe and even larger proportions of other micronutrients, are not reallocated to seeds (**Fig.4**; Guiboileau et al., 2012). Thus, increasing further autophagy process specifically during seed filling may be a powerful approach to increase the pool of nutrients available for subsequent translocation to seeds. However, autophagy is a complex mechanism subjected to a subtle multiscale regulation (Yoshimoto, 2012; Masclaux-Daubresse et al., 2017). Further investigations on the regulation of autophagy may thus help improve grain yield by bioengineering strategies or marker assisted breeding.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) ecotype Colombia-0 (Col-0) [*atg5-1* (SALK_020601), *atg9-2* (SALK_130796), AtATG18a RNAi (RNAi18), *sid2*, *atg5sid2*], and ecotype Wassilewskija (WS) [*atg4* (*atg4a4b-1*), *atg9-1*] have been described previously (Hanaoka et al., 2002; Yoshimoto et al., 2004; Xiong et al., 2005; Inoue et al., 2006; Yoshimoto et al., 2009).

The first experiment including all autophagy deficient plants presented in this study was conducted on sand. Seeds were sawn on sand and plants were watered three times per week for 2 hours with high nitrogen nutritive solution as described in Chardon *et al.*(2010). Plants were grown under the following conditions: 8/16 h 21/17°C light/dark until 56 days after sowing (DAS) and then transferred to long days (16 h light), maintaining similar day/night temperatures. When plants were dry, seeds were separated from dry remains of vegetative tissues, including leaves, stems, and siliques to determine their respective dry weights and metal contents.

In other experiments performed to determine the involvement of autophagy in Fe remobilization, plants were cultivated on a 1/1 mix of sand and perlite, watered three times per week for 2 hours with modified Hoagland solution [0.28 mM KH_2PO_4, 1.25 mM KNO_3, 0.75 mM MgSO4, 1.5 mM $Ca(NO_3)$, 25 µM H_3BO_3, 50 µM KCl, 1 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 0.5 µM $CuSO_4$, 5 µM $MnSO_4$, and 3 mM MES-KOH, pH 5.7]. Ten micromolar Fe was provided as Fe^{3+} chelated to HBED [N,N'-di(2-hydroxybenzyl) ethylene diamine-N,N'-

diacetic acid monochloride hydrate; Strem Chemicals, Newburyport, Massachusetts, USA] prepared as described by Lanquar *et al.* (2005). Plants were grown in a climate chamber under the following conditions: 9/15 h, 21/19°C light/dark; light enriched in blue and red wavelengths (OSRAM FLUORA, Munich, Germany); light intensity of 100 µmol of photon.m^{-2}.s^{-1}; 75% relative humidity until 68 days after sowing. Then, plants were transferred to long days (16 h light), maintaining similar conditions. At the onset of flowering (78 DAS) half of plants were maintained in Fe sufficient nutrition (solution containing 10 µM Fe-HBED) and the other half were transferred to Fe deprivation nutrition (solution containing 20 µM of ferrozine) until the end of the plant life cycle. When plants were dry, leaves, seeds, stems, and empty siliques were harvested separately to determine their dry weight and their metal content. The experiment presented in supplemental data was performed in the same condition, excepted that white light was provided by TLD 58W 830 and 840 (Philips, Amsterdam, Netherlands) with an intensity of 150 µmol of photon.m^{-2}.s^{-1}.

^{57}Fe-labeling

For ^{57}Fe labeling, $^{57}Fe^{3+}$ (96.28 Atom %) was prepared and combined with HBED to replace ^{56}FeHBED by ^{57}FeHBED in the modified Hoagland solution. After four days without watering, plants were placed in presence of ^{57}FeHBED modified Hoagland solution for 24 h precisely, at 52 and 54 DAS, during the vegetative phase. After labeling, the pots containing substrate and roots were rinsed four times with MilliQ water and one time with non-labeled solution to discard ^{57}Fe. Then, unlabeled modified Hoagland solution was used for the rest of the culture cycle until seed harvest.

Metal concentration analysis

After weight measurement, dried samples were digested in 2 mL of 70% nitric acid in a DigiBlock ED36 (LabTech, Italy) at 100°C for 1 h, 120°C for 6 h and 80°C for 1 h. After dilution in trace-metal-free water, the metal content of the samples was determined by atomic absorption spectrometry using an AA240FS flame spectrometer or a MP AES 4200 Atomic Emission spectrometer (Agilent, USA).

Determination of ^{57}Fe/^{56}Fe ratios and remobilization indices

An aliquot of 50 mg of dried and crushed sample was digested in a mixture of nitric acid hydrogen peroxide (ultra-pure reagent grade) on a heated block (DigiPrep (SCP Science,

Canada) at 90°C during 3 hours and then diluted to 50 mL with milliQ water. Blank samples are prepared in the same conditions.

The measurement of the isotopes ^{56}Fe and ^{57}Fe contained in these solutions was performed using inductively coupled plasma mass spectrometry, ICP MS (Perkin Elmer Nexion, USA). Due to the specific polyatomic interferences caused by argon and calcium oxides, helium gas was introduced in the dynamic collision cell to prevent from the specific polyatomic interferences caused by argon and calcium oxides. Quality control solutions were prepared from a standard solution containing 100 $mg.mL^{-1}$ of natural sourced iron (Inorganic Ventures) and placed during the whole analytical sequence.

Statistical analysis

Experiments were carried out in three to eight independent biological replicates. Data were analyzed with Kruskal-Wallis and Mann-Whitney non-parametric tests for multiple comparisons and pair comparisons, respectively. For multiple comparisons, a Tukey post hoc test was performed when significant differences were detected ($p < 0.05$). Different letters indicate significant differences between samples. All tests were performed using the R software package.

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Figure Legends

Figure 1. Defects in autophagy lead to elevated Zn, Mn, and Fe concentrations in vegetative tissues of *Arabidopsis thaliana*. Zn (A), Mn (B) and Fe (C) concentrations in dry remains of vegetative tissues of wild-type plants, (Wassilewskija and Colombia-0; black bars) and autophagy deficient plants (gray bars) in the same genetic backgrounds, grown on sand as described in Chardon *et al.* (2010). Results are shown as mean ± SE of four biological repeats. Different letters indicate significant differences between genotypes according to a Kruskal-Wallis test ($p < 0.05$, n = 4) followed by a Tukey *post hoc* test; *n.s.d.* indicates no significant difference.

Figure 2. Distinct mechanisms lead to decrease in Fe concentration in seeds of autophagy deficient plant depending on Fe supply during the reproductive stage in *Arabidopsis thaliana*. Fe concentrations in leaves, stems (including empty siliques) and seeds of wild-type (WT, black bars), *atg5-1* mutant (light grey bars), *atg5sid2* double mutant (brown bars) and *sid2* mutant plants grown on sand/perlite (1/1) substrate watered with modified Hoagland medium. Iron was supplied (Fe sufficient) or not (Fe deprivation) during the reproductive stage. Results are shown as mean ± SE of five biological repeats. Different letters indicate significant differences according to a Kruskal-Wallis test ($p < 0.05$, n = 5) followed by a Tukey *post hoc* test.

Figure 3. Total Fe content, Fe distribution, and Fe translocation to seeds are dramatically affected in *atg5-1* and *atg5sid2* mutants of *Arabidopsis thaliana*. A, Distribution of Fe between leaves (closed squares), stems including empty siliques (diagonal stripes), and seeds (white dots) of plants are represented for the wild-type (WT, black bars), the *atg5-1* (light gray bars), the *atg5sid2* (brown bars), and the *sid2* (dark gray) mutant plants. B, total Fe translocation efficiency calculated as the ratio between the Fe content in seeds and the Fe content in the whole plant (Fe_{seed} / (Fe_{leaf} + Fe_{stem} + Fe_{seed}). C, ^{57}Fe translocation efficiency (remobilized Fe translocation efficiency) calculated as $^{57}Fe_{seed}$ / ($^{57}Fe_{leaf}$ + $^{57}Fe_{stem}$ + $^{57}Fe_{seed}$) after ^{57}Fe pulse labeling at the vegetative stage. Plants were grown on sand/perlite (1/1) substrate watered with modified Hoagland medium. Fe was supplied (Fe sufficient) or not (Fe deprivation) during the reproductive stage. Results are shown as mean ± SE of five biological repeats. Different letters indicate significant differences between genotypes and conditions according to a Kruskal-Wallis test ($p < 0.05$, n = 5) followed by a Tukey *post hoc* test.

Figure 4. Translocation efficiencies of Zn (A) and Mn (B) are also affected in *atg5-1* and *atg5sid2* mutant plants of *Arabidopsis thaliana*. Translocation efficiencies were calculated for wild-type (WT), *atg5-1*, *atg5sid2*, and *sid2* mutant plants, as the ratio between the micronutrient content in seeds and the micronutrient content in the whole plant. Plants were grown on sand/perlite (1/1) substrate watered with modified Hoagland medium containing Fe during the whole plant cycle. Results are shown as mean ± SE of five biological replicates. Different letters indicate significant differences between genotypes according to a Kruskal-Wallis test ($p < 0.05$, n = 5) followed by a Tukey *post hoc* test.

Figure 5. Iron fluxes in wild-type *Arabidopsis thaliana* growing under Fe sufficient condition, in vegetative, early reproductive and late reproductive stages, base of results obtained in wild-type and autophagy deficient plants. Thicknesses of the uptake and remobilization arrows are

proportional to flux proportions. Iron distribution (brown circles), are expressed as percentage of total iron of wild-type plants harvested after completion of the life cycle. Early reproductive stage is defined as the period of premature senescence in *atg5-1*. Late reproductive stage is defined as the period of senescence in the wild-type, when *atg5-1* mutant is already dry. Together, the results obtained indicate that (1) most of the iron uptake (71±4%) takes place during the vegetative growth, (2) iron uptake after the onset of flowering (29±5%) occurs mostly during the early reproductive stage, (3) seed loading is mainly achieved through remobilization, rather than direct contribution from Fe root uptake, (4) in wild-type 35% the total iron content is loaded to seeds through autophagy dependent remobilization, and 19 % (54% - 35%) is loaded through autophagy independent remobilization.

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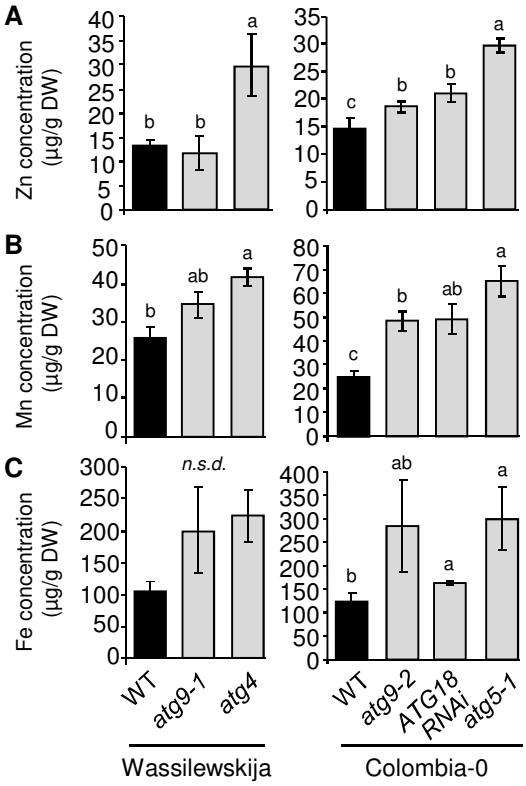

Figure 1. Defects in autophagy lead to elevated Zn, Mn, and Fe concentrations in vegetative tissues of *Arabidopsis thaliana*. Zn (A), Mn (B) and Fe (C) concentrations in dry remains of vegetative tissues of wild-type plants, (Wassilewskija and Colombia-0; black bars) and autophagy deficient plants (gray bars) in the same genetic backgrounds, grown on sand as described in Chardon *et al.* (2010). Results are shown as mean ± SE of four biological repeats. Different letters indicate significant differences between genotypes according to a Kruskal-Wallis test ($p < 0.05$, n = 4) followed by a Tukey *post hoc* test; *n.s.d.* indicates no significant difference.

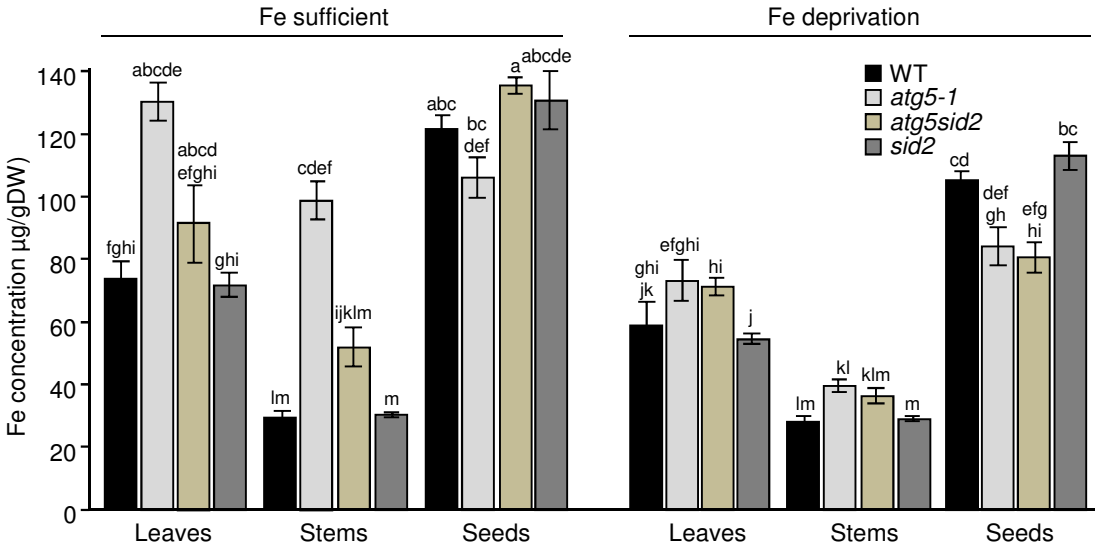

Figure 2. Distinct mechanisms lead to decrease in Fe concentration in seeds of autophagy deficient plant depending on Fe supply during the reproductive stage in *Arabidopsis thaliana*. Fe concentrations in leaves, stems (including empty siliques) and seeds of wild-type (WT, black bars), *atg5-1* mutant (light grey bars), *atg5sid2* double mutant (brown bars) and *sid2* mutant plants grown on sand/perlite (1/1) substrate watered with modified Hoagland medium. Iron was supplied (Fe sufficient) or not (Fe deprivation) during the reproductive stage. Results are shown as mean ± SE of five biological repeats. Different letters indicate significant differences according to a Kruskal-Wallis test ($p < 0.05$, n = 5) followed by a Tukey *post hoc* test.

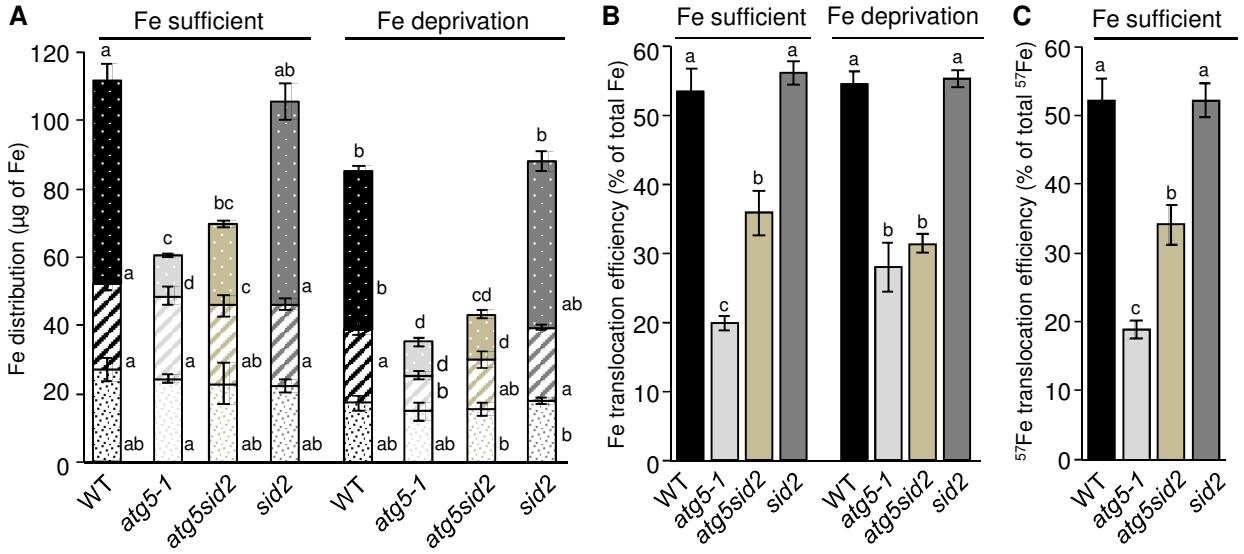

Figure 3. Total Fe content, Fe distribution, and Fe translocation to seeds are dramatically affected in *atg5-1* and *atg5sid2* mutants of *Arabidopsis thaliana*. A, Distribution of Fe between leaves (closed squares), stems including empty siliques (diagonal stripes), and seeds (white dots) of plants are represented for the wild-type (WT, black bars), the *atg5-1* (light gray bars), the *atg5sid2* (brown bars), and the *sid2* (dark gray) mutant plants. B, total Fe translocation efficiency calculated as the ratio between the Fe content in seeds and the Fe content in the whole plant (Fe_{seed} / (Fe_{leaf} + Fe_{stem} + Fe_{seed}). C, ^{57}Fe translocation efficiency (remobilized Fe translocation efficiency) calculated as ($^{57}Fe_{seed}$ / ($^{57}Fe_{leaf}$ + $^{57}Fe_{stem}$ + $^{57}Fe_{seed}$) after ^{57}Fe pulse labelling at the vegetative stage. Plants were grown on sand/perlite (1/1) substrate watered with modified Hoagland medium. Fe was supplied (Fe sufficient) or not (Fe deprivation) during the reproductive stage. Results are shown as mean ± SE of five biological repeats. Different letters indicate significant differences between genotypes and conditions according to a Kruskal-Wallis test ($p < 0.05$, n = 5) followed by a Tukey *post hoc* test.

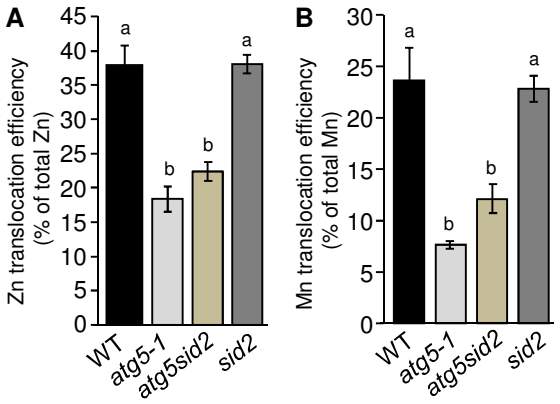

Figure 4. Translocation efficiencies of Zn (A) and Mn (B) are also affected in *atg5-1* and *atg5sid2* mutant plants of *Arabidopsis thaliana*. Translocation efficiencies were calculated for wild-type (WT), *atg5-1*, *atg5sid2*, and *sid2* mutant plants, as the ratio between the micronutrient content in seeds and the micronutrient content in the whole plant. Plants were grown on sand/perlite (1/1) substrate watered with modified Hoagland medium containing Fe during the whole plant cycle. Results are shown as mean ± SE of five biological replicates. Different letters indicate significant differences between genotypes according to a Kruskal-Wallis test ($p < 0.05$, n = 4) followed by a Tukey *post hoc* test.

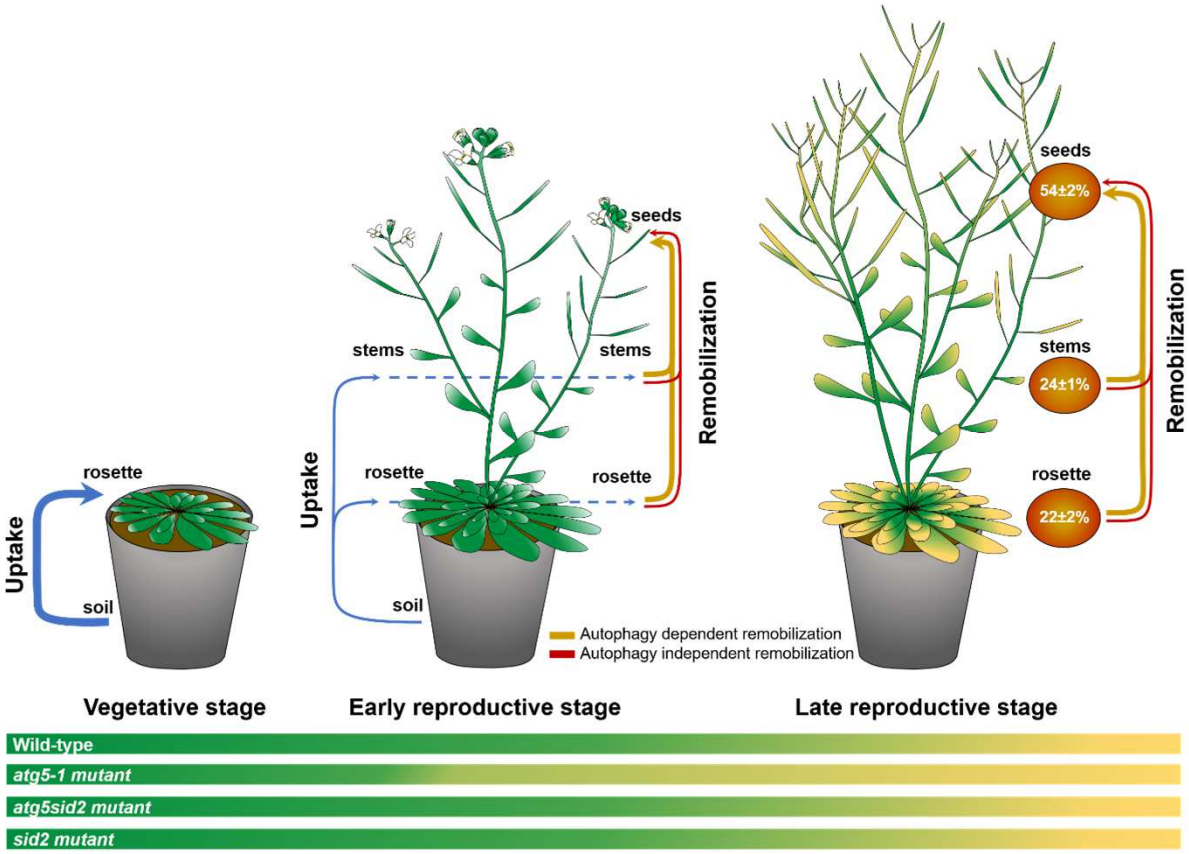

Figure 5. Iron fluxes in wild-type *Arabidopsis thaliana* growing under Fe sufficient condition, in vegetative, early reproductive and late reproductive stages, base of results obtained in wild-type and autophagy deficient plants. Thicknesses of the uptake and remobilization arrows are proportional to flux proportions. Iron distribution (brown circles), are expressed as percentage of total iron of wild-type plants harvested after completion of the life cycle. Early reproductive stage is defined as the period of premature senescence in *atg5-1*. Late reproductive stage is defined as the period of senescence in the wild-type, when *atg5-1* mutant is already dry.
Together, the results obtained indicate that (1) most of the iron uptake (71±4%) takes place during the vegetative growth, (2) iron uptake after the onset of flowering (29±5%) occurs mostly during the early reproductive stage, (3) seed loading is mainly achieved through remobilization, rather than direct contribution from Fe root uptake, (4) in wild-type 35% the total iron content is loaded to seeds through autophagy dependent remobilization, and 19 % (54% - 35%) is loaded through autophagy independent remobilization.