RESEARCH PAPER

Increases in activity of proteasome and papain-like cysteine protease in Arabidopsis autophagy mutants: back-up compensatory effect or cell-death promoting effect?

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Abstract

Autophagy is essential for protein degradation, nutrient recycling, and nitrogen remobilization. Autophagy is induced during leaf ageing and in response to nitrogen starvation, and is known to play a fundamental role in nutrient recycling for remobilization and seed filling. Accordingly, ageing leaves of Arabidopsis autophagy mutants (atg) have been shown to over-accumulate proteins and peptides, possibly because of a reduced protein degradation capacity. Surprisingly, atg leaves also displayed higher protease activities. The work reported here aimed at identifying the nature of the proteases and protease activities that accumulated differentially (higher or lower) in the atg mutants. Protease identification was performed using shotgun LC-MS/MS proteome analyses and activity-based protein profiling (ABPP). The results showed that the chloroplast FTSH (FILAMENTATION TEMPERATURE SENSITIVE H) and DEG (DEGRADATION OF PERIPLASMIC PROTEINS) proteases and several extracellular serine proteases [subtilases (SBTs) and serine carboxypeptidase-like (SCPL) proteases] were less abundant in atg5 mutants. By contrast, proteasome-related proteins and cytosolic or vacuole cysteine proteases were more abundant in atg5 mutants. Rubisco degradation assays and ABPP showed that the activities of proteasome and papain-like cysteine protease were increased in atg5 mutants. Whether these proteases play a back-up role in nutrient recycling and remobilization in atg mutants or act to promote cell death is discussed in relation to their accumulation patterns in the atg5 mutant compared with the salicylic acid-depleted atg5/sid2 double-mutant, and in low nitrate compared with high nitrate conditions. Several of the proteins identified are indeed known as senescence- and stress-related proteases or as spontaneous cell-death triggering factors.

Keywords: AALP, RD21, CATHB3, SAG12, metacaspase, nitrogen remobilization, senescence.
Introduction

Autophagy is a universal mechanism that facilitates the degradation of unwanted cell constituents in the lytic compartments of eukaryotic cells. Autophagy is essential for the recycling of cellular material and controls nitrogen remobilization and grain-filling in Arabidopsis (Guiboileau et al., 2012; Masclaux-Daubresse et al., 2017). The autophagy machinery consists of the formation of cytosolic double-membrane vesicles, termed autophagosomes, that engulf and sequester unwanted cytoplasmic constituents such as damaged organelles and protein aggregates (Liu and Bassham, 2012). Degradation does not occur directly inside the autophagosome as molecules destined for recycling are transported to the lytic vacuoles where proteases and hydrolases operate. Autophagosome formation involves the products of AUTOPHAGY (ATG) genes, previously discovered in yeast by the pioneering work of Professor Yoshinori Ohsumi (Nobel Prize in Medicine or Physiology 2016; Tsukada and Ohsumi, 1993). Subsequently, homologous genes in plants and animals have been found for almost all ATGs. Among the 50 ATG genes discovered in yeast, 18 are part of the autophagy core machinery (Yang and Bassham, 2015). These genes are absolutely essential to the formation of autophagosomes. In Arabidopsis, the core machinery genes are either single or belong to gene families. In particular, ATG5, involved in the ATG5–ATG12 conjugation system, is essential for the formation of the ATG8-PE (PE, phosphatidylethanolamine) conjugate that features on the autophagosome membrane and helps the expansion of the vesicle membrane (Masclaux-Daubresse et al., 2017). The atg5 Arabidopsis mutants have been intensively studied (Thompson et al., 2005; Yoshimoto et al., 2009; Lenz et al., 2011; Guiboileau et al., 2012, 2013; Masclaux-Daubresse et al., 2014; Floyd et al., 2015; Wang et al., 2015; Fahy et al., 2017). Like the other atg mutants studied so far (atg9, atg7, and atg2), the atg5 mutants present smaller rosettes, hypersensitivity to N and C starvation, and reduced yield. Using 13N-tracer experiments, Guiboileau et al. (2012) showed that the atg5 and atg9 mutants and the atg18a-RNAi line are strongly affected in N remobilization, and we have verified further that this is also the case for many other atg single-mutants (C. Masclaux-Daubresse, unpublished results). Several lines of evidence show that N and C leaf metabolisms are strongly affected in atg5, atg9, and atg18a-RNAi (Guiboileau et al., 2013; Masclaux-Daubresse et al., 2014). Mutants have lower starch, sucrose, and hexose contents in their leaves and higher amounts of amino acids (especially glutamate, aspartate, and methionine). They also display higher protein and RNA concentrations relative to wild-type plants. In order to try and explain such phenotypes, Guiboileau et al. (2013) measured the endo-, carboxy-, and amino-peptidase activities, and a series of western blots were also carried out in order to determine whether protein accumulation was selective. The results showed that several proteins and peptides were specifically accumulated in the leaves of atg mutants. The absence of transcriptional changes suggested that these proteins and peptides were specific cargoes. Surprisingly, protease activities were significantly increased in the leaves of atg mutants (Guiboileau et al., 2013), showing that the accumulation of the peptides and proteins over-abundant in the atg rosettes was not related to lower proteolysis capacity. In was then hypothesized that in the absence of autophagosome trafficking, vacuole proteases and their cytoplasmic substrates cannot co-localize, resulting in protein over-accumulation.

The aim of this study was to identify which protease activities are increased in autophagy mutants in order to compensate for the defects in autophagy-dependent protein recycling and to provide alternative remobilization pathways. Proteomics shotgun LC-MS/MS analyses were carried out in order to identify the proteases and proteins with increased or decreased accumulation in atg5. Because part of the phenotypes of autophagy mutants is related to the increase of salicylic acid (SA) in atg leaves, the atg5/sid2 double-mutant was also analysed in order to discriminate SA-dependent and SA-independent changes. As a low-nitrate regime is known to promote nitrogen remobilization and autophagy activity, mutants were grown under both low- and high-nitrate conditions. Low-nitrate is indeed more favourable for the detection of autophagy-related phenotypes (Guiboileau et al., 2012, 2013; Masclaux-Daubresse et al., 2014). In addition to the identification of the proteases differentially accumulated in atg mutants, we monitored whether they were active by using specific probes (Morimoto and van der Hoorn, 2016), and performed western blots in order to evaluate the respective amounts of their pro- and active-forms. The results are discussed with regards to the known relationships that exist between protease activities, leaf senescence, N remobilization, and cell death. This work reveals new candidates that may participate in the management of N remobilization and possibly in the last steps of autophagy in Arabidopsis leaves.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Columbia wild-type, atg5 (SALK_020601), atg5/sid2, and sid2 mutants have been previously characterized by Yoshimoto et al. (2009) and Guiboileau et al. (2012, 2013). Plants were cultivated according to Guiboileau et al. (2013) under low- (LN; 2 mM) and high- (HN; 10 mM) nitrate conditions. Adequate growth conditions for the testing of nitrogen-limitation effects on Arabidopsis plants have previously been established by L oudet et al. (2003a, 2003b) and further improved by Lemaitre et al. (2008), (see Supplementary Method S1 at JXB online for details). Whole rosettes were harvested at 60 d after sowing (DAS). Three independent plant replicates were sampled. For HN, each biological replicate contained four rosettes; for the LN, each biological replicate contained 24 rosettes. Harvests commenced 2 h into the 8-h photoperiod and were completed within 1 h. Samples were stored at −80 °C for subsequent use. Three batches of plants were cultured, providing samples from three independent experiments. The analyses detailed below were then performed on three biological replicates and
repeated on samples from 2–3 independent batches of plants. For validation of the anti-RD21A antibody, we used the homozygous rd21a SALK_090550C T-DNA insertion mutant (Wang et al., 2008). For validation of the anti-CATHB3 antibody, the SALK_19630 cathb3 homozygous T-DNA insertion mutant was used (Ge et al., 2016).

**Shotgun proteomic analysis**

Leaf total proteins were extracted, digested, and analysed by LC-MS/MS using the PAPSSO platform (INRA, Le Moulon, Gif sur Yvette; see Supplementary Method S2 for details). Protein identification was performed using X! Tandem Piledriver (version 2015.04.01; http://www.thegpm.org/TANDEM/index.html) by querying the MS/MS data against the TAIR10 protein library together with a custom contaminant database (trypsin, keratins). Identified proteins were filtered and grouped using X! Tandem Pipeline (3.4.1; pappso.inra.fr/bioinfo/xstandempipeline/) (Langella et al., 2017) according to two criteria: (1) a minimum of two different peptides required with an E-value smaller than 0.01, and (2) a protein E-value (calculated as the product of unique peptide E-values) smaller than 10^{-5}. The false discovery rates (FDRs) at the peptide and protein levels were 0.03% and 0.0%, respectively. Relative peptide quantification by peak area integration on extracted ion chromatogram (XICs) was performed using the MassChroQ software (pappso.inra.fr/bioinfo/masschroq/) (Valot et al., 2011). Relative protein abundance was calculated and defined as the sum of peptide intensities considering only (1) reproducible peptides, (2) specific peptides, and (3) correlated peptides belonging to the same protein (see Supplementary Method S2 for details). When the peptides of a protein were not present or not reproducibly observed in one or more conditions, spectral counting (SC) was used in place of XICs analysis.

**Activity-based protein profiling of papain-like cysteine proteases, proteasome and serine proteases**

Papain-like cysteine proteases (PLCPs), serine proteases, and proteasome activity profiling were assayed using DCG-04 (van der Hoorn et al., 2004), desthiobiotin-FP (DFP; Thermo Scientific) and MVB072 (Kolodziejek et al., 2011), respectively. DCG-04 was synthesized as described by Greenbaum et al. (2000). The protein extract obtained by homogenizing 200 mg of frozen material with 450 µl of water (1.5% polyvinylpyrrolidone, w/v) was centrifuged (20 000 × g, 15 min, 4 °C). Protein concentration was determined using the 2D Quant kit (SIGMA-ALDRICH). The elution step was repeated on samples from 2–3 independent batches of plants. For validation of the anti-RD21A antibody, we used the homozygous rd21a SALK_090550C T-DNA insertion mutant (Wang et al., 2008). For validation of the anti-CATHB3 antibody, the SALK_19630 cathb3 homozygous T-DNA insertion mutant was used (Ge et al., 2016).

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Affinity purification and identification of PLCPs

Only plants grown under low-N were analysed. For each genotype, soluble proteins from four independent rosette bulk samples were extracted as described above. An equal amount of proteins (750 µg) from first and second, and from third and fourth bulk samples were mixed to obtain two mixed protein solutions per genotype. Then, 1.5 mg of proteins were labelled with 50 mM NaAc, pH 5.5, 2 mM DTT, and 5 mM DCG-04 in 1 ml of reaction volume and incubated for 150 min at room temperature in the dark. Labelling reactions were stopped and the biotinylated proteins were purified as described by Chandrasekar et al. (2014), except that avidin beads were used (SIGMA-ALDRICH). The elution step was repeated once and the two eluates were mixed and separated by 12% SDS-PAGE. Gels were stained with silver nitrate and bands with a molecular mass between 25 and 40 kDa, corresponding to bands observed in the activity-based protein profiling (ABPP) experiment, were excised with a OneTouch GridCutter (2.0 × 7.0 mm; Gel Company). Each slice was placed into a 0.5-ml volume containing 100 µl of 4× SDS-PAGE loading buffer and resolved by 12% or 15% SDS-PAGE. Resolved MVB072-labeled proteins were visualized by in-gel fluorescence scanning using a Typhoon 9400 scanner (GE Healthcare Life Science) with excitation and emission wavelengths at 532 and 580 nm, respectively. Resolved DCG-04- and desthiobiotin-FP-labelled proteins were transferred to a polyvinylidene fluoride membrane. Membranes were incubated overnight in 1% BSA (w/v) in PBS-T 0.1% and incubated with streptavidin-HRP (Ultrasonic) for 1 h. The chemiluminescence signal was visualized using an ImageQuant LAS 4000 (GE Healthcare Life Science). Colloidal Blue-stained gels were used to monitor protein loading. Fluorescence, chemiluminescence, and Coomassie signals were quantified using the ImageQuant software (GE Healthcare Life Science).

**Western blots**

Total proteins were extracted by homogenizing 100 mg of frozen material in 500 µl of buffer [7 M urea, 2 M thiourea,
30 mM Tris-HCl pH 8.8, 4% CHAPS (w/v), 0.2% triton X100 (w/v), 20 mM DTT, and 1× Complete protease inhibitor cocktail (Roche). Experiments were centrifuged and protein concentration was determined as described above. For each sample, 5 µg of proteins was separated on 12% SDS-PAGE. Resolved proteins were electroblotted (Trans-Blot Turbo transfer system; Bio-Rad). Polypeptide detections were performed using antibodies raised against the RD21A-specific peptide DELPESIDWRKKG, against SAG12 (Agrisera, Vännäs, Sweden), and against the N-terminal end of the CATHB3 mature protein (synthetic peptide LPKAFDARTA WPQC). Primary antibodies were diluted 1:1000 in 5% milk PBS-T 0.1% buffer for membrane incubation. Secondary HRP peroxidase antibodies (1:10000) were used for chemiluminescence detection. Signals were detected using an ImageQuant LAS 4000 (GE Healthcare Life Science). Coomassie Brilliant Blue-stained gels were used to verify that an equal amount of protein was loaded in each lane. RD21A and CATHB3 antibody specificities were tested using rd21A and cathb3 mutants as negative controls (see Supplementary Fig. S1).

Bioinformatics and statistical analyses

All statistical analyses were performed using the R software (https://www.r-project.org/). Data were analysed using two-way ANOVA with genotype and nutrition conditions as variable factors, followed by a Tukey’s post-hoc test. For the relative quantification of proteins by the XICs method, statistical analyses were carried out on log2-transformed protein abundance. Proteins corresponding to proteases with a Tukey’s P-value ≤0.05 were considered to be significantly differentially accumulated. Fold-change (FC) ratios were determined as abundance ratios of atg5 versus Col and of atg5/sid2 versus sid2. No FC threshold was applied as even a small change in protease abundance can have a strong impact on the proteome due to post-translational regulation and the large substrate spectrum of proteases. In order to identify proteases among the proteins detected by LC-MS/MS, Gene Ontology and functional information for each protein were analysed using the following databases: MEROPS (https://www.ebi.ac.uk/merpoms/), UniProt (www.uniprot.org/), agriGO (bioinfo.cau.edu.cn/agriGO), and Mapman (mapman.gabipd.org/).

Accession numbers

| AT4G38220 | AT2G27020 | AT2G4200 | AT4G20850 |
| AT5G35590 | AT4G31300 | AT3G22110 | AT2G05840 |
| AT4G14800 | AT5G36082 | AT1G53750 | AT3G05530 |
| AT4G17510 | AT5G05780 | AT5G58290 | AT5G10540 |
| AT1G21720 | AT1G56450 | AT5G42790 | AT4G01610 |
| AT1G47128 | AT1G38380 | AT5G45890 | AT5G51070 |
| AT3G13235 | AT1G79340 | AT1G50380 | AT3G15260 |
| AT5G23540 | AT4G38630 | AT1G51710 | AT5G66140 |
| AT4G39010 | AT4G39090 | AT5G43060 | AT5G10760 |
| AT1G16470 | AT1G79210 | AT2G14260 | AT5G23140 |
| AT4G16190 | AT5G26860 | AT3G14290 | AT3G20630 |
| AT2G41790 | AT4G36760 | AT5G36210 | AT1G50250 |
| AT5G42270 | AT4G36195 | AT1G09750 | AT3G54400 |
| AT3G02110 | AT2G39850 | AT1G52510 | AT2G47940 |
| AT2G35780 | AT5G39830 | AT3G27925 | AT3G14067 |
| AT3G19170 | AT4G24390 | AT3G24590 | AT1G13270 |
| AT5G05740 | AT2G30950 | AT4G18370 | AT3G52500 |
| AT5G42240 | AT1G06430 | AT5G23210 | AT3G05350 |
| AT5G65760 | AT1G067700 | AT3G61820 | AT1G01300 |
| AT4G34980 | AT2G05920 | AT3G18490 | AT2G33530 |
| AT4G21650 | AT5G08260 | AT1G09130 | AT1G21750 |
| AT1G47710 | AT4G16500 | AT517290 | AT5G60360 |
| AT5G50260 | AT1G02305 | AT4G35350 |

Results

LC-MS/MS identification of protease proteins in autophagy mutants

Shotgun LC-MS/MS proteome analyses were carried out to identify the nature of the proteomes specifically accumulated in atg5 mutants. Total proteins were extracted from the autophagy-defective lines (atg5 and atg5/sid2) and their respective controls (Col and sid2) grown for 60 d under high-N (HN) and low-N (LN) as previously described by Guiboileau et al. (2013). The atg5/sid2 double-mutant was analysed in addition to atg5 in order to discriminate against the side effects of salicylic acid (SA) (Guiboileau et al., 2012). With regards to previous studies (Yoshimoto et al., 2009; Guiboileau et al., 2012, 2013; Masclaux-Daubresse et al., 2014), we preferred here to consider atg5/sid2 versus sid2 rather than atg5/NahG versus NahG in order to discriminate against SA effects because of the lower SA content found in the sid2 background relative to the NahG background (see Supplementary Fig. S2). The rosette phenotypes at harvest are shown in Fig. 1a, b. Under LN conditions, no leaf senescence phenotype was observed in any of the four genotypes (Fig.1a). Under LN conditions, a slight senescence phenotype was observed on the oldest leaves of atg5; this phenotype was mitigated in atg5/sid2. In accordance with Guiboileau et al. (2013), we confirmed that the total protein concentration was higher in atg5 relative to the Col wild-type under LN, and significantly higher in atg5/sid2 compared to sid2 under both LN and HN (Fig.1c).

LC-MS/MS shotgun proteome analyses were then carried out on the total protein extracted from three independent biological repeats. XICs analysis permitted the relative quantification of proteins when peptides were detected in all the genotypes. When a peptide was undetectable in one of the two genotypes under comparison, individual peptide quantification by spectral count (SC) was performed. XICs and SC analyses, respectively, identified increased accumulation of 47 and decreased accumulation of 36 protease-related proteins in the autophagy-defective lines relative to control lines (Tables 1 and 2).

The predicted localization at subcellular level of the proteases and protease-related proteins that either increased or decreased in accumulation in the autophagy-deficient lines...
are shown in Fig. 2a. Among the 47 protease-related proteins that increased in autophagy mutants, we found mainly proteasome subunits (22 proteins; Fig. 2b) and cytosol-localized proteases (28% of 26 proteases; Fig. 2a). Proteasome subunits were both core proteases (α and β sub-units: 50% and 23%, respectively; Fig. 2b) and regulatory particles (14% for both base and lid complexes). Vacuole-, plastid-, and extracellular-predicted proteases were also identified (17%, 16%, and 14%, respectively; Fig. 2a). Accumulated proteases were mostly cysteine (38%), serine (27%), and metal proteases (23%) (Fig. 2c).

The 36 proteases that had decreased accumulation were mainly plastid-predicted (44%) and extracellular-predicted proteases (42%), and they belong to the serine (55%), metallo- (28%) and, to a lesser extent, aspartate (17%) proteases. There was no cysteine proteases or proteasome sub-units among the decreased proteins.

Venn diagrams of the proteases and proteasome subunits that were significantly differentially accumulated in autophagy-deficient lines are presented in Fig. 3. Among the proteases that were accumulated compared to the controls (Table 1; Fig. 3a), seven were more abundant in both atg5 and atg5/sid2, and under both nitrate conditions (Class1-up), four proteases were more abundant in atg5/sid2 under both LN and HN, and also in atg5 under LN (Class2-up), and 12 were specifically over-accumulated under LN in both atg5 and atg5/sid2, i.e. in a SA-independent manner (Class3-up). The majority of the Class1-up, 2-up, and 3-up proteases were part of the proteasome machinery (Table 1), either threonine proteases or regulatory sub-units of the proteasome. Several proteasome-associated proteases such as LAP1, TOP2, and TPP2, or protein deubiquitinases such as UCH3 were also identified (Polge et al., 2009). In Class1-up, only AQI (AQUAPORIN INTERACTOR) was not related to the proteasome, and in Class3-up, three well-known cysteine proteases CATHB3 (CATHEPSIN B3), RD21A (RESPONSIVE TO DEHYDRATION 21A), and SAG12 (SENESCENCE ASSOCIATED GENE 12) were found. Class4-up included proteases significantly accumulated compared to the controls in atg5 only (i.e. SA-dependent) and under both LN and HN. It included the chloroplast CLPD subunit (also referred to as SAG15 or ERD1), the Arabidopsis METACASPASE 4 (AMC4), the ubiquitin-family aspartate protease DDI1 (DNA-DAMAGE INDUCIBLE 1), which is induced under cadmium stress, and an unknown serine protease (At1g50380). The proteins of Class5-up were specifically over-accumulated in atg5 under LN. Class5-up included two proteasome non-catalytic sub-units (PAD1 and PAD2), two proteasome regulatory proteins (RPN10 and RPN11), the deubiquitinase UBP6, the leucine aminopeptidase LAP3, the two drought resistance-related cysteine proteases RD19A and RD21B, and the AED1 (APOPLASTIC, EDS1-DEPENDENT) aspartate protease. Class6-up, which represents proteases over-accumulated in atg5/sid2 under high N, included PIP (PROLINE IMINOPEPTIDASE) and the two proteasome subunits PAB1 and PAB2. Class7-up
### Table 1. List of the proteases with significantly increased accumulation in atg5 versus Col, and in atg5/sid2 versus sid2

| M(1) | Accession (TAIR10) | Name(2) | Catalytic class(3) | Predicted subcellular localization(4) | Fold-change(5) | atg5 | atg5/sid2 |
|------|---------------------|---------|-------------------|--------------------------------------|----------------|------|----------|
| Class 1 | XIcs AT4G38220 | AQI | Met | ER | 1.63 | 1.77 | 1.84 | 2.21 |
| XIcs AT2G27020 | PAG1 Thr | 20S (CP α) | 1.41 | 1.79 | 1.64 | 1.84 |
| XIcs AT2G24200 | LAP1 Met | PM,C | 1.44 | 1.55 | 1.42 | 1.41 |
| XIcs AT4G20850 | TPP2 Ser | Pl | 1.38 | 1.48 | 1.38 | 1.55 |
| XIcs AT5G35590 | PAA1 Thr | 20S (CP α) | 1.24 | 1.58 | 1.28 | 1.63 |
| XIcs AT4G31300 | PBA1 Thr | 20S (CP β) | 1.33 | 1.37 | 1.40 | 1.41 |
| XIcs AT3G22110 | PAC1 Thr | 20S (CP α) | 1.28 | 1.51 | 1.20 | 1.46 |
| Class 2 | XIcs AT2G05840 | PAA2 Thr | 20S (CP α) | 1.28 | 1.73 | 1.43 | 1.72 |
| XIcs AT4G14800 | PBD2 Thr | 20S (CP β) | 1.19 | 1.80 | 1.43 | 1.65 |
| XIcs AT3G60820 | PBF1 Thr | 20S (CP β) | 1.15 | 1.49 | 1.23 | 1.52 |
| XIcs AT1G53750 | RPT1A non-catalytic | 19S (RP base) | 1.25 | 1.42 | 1.29 | 1.47 |
| Class 3 | XIcs AT3G05530 | RPT5A non-catalytic | 19S (RP base) | 1.31 | 2.29 | 1.39 | 2.33 |
| XIcs AT4G17510 | UCH3 Cys | C | 1.55 | 2.27 | 1.82 | 2.09 |
| XIcs AT5G05780 | RPN8A non-catalytic | 19S (RP lid) | 1.18 | 2.44 | 1.31 | 1.88 |
| XIcs AT5G58290 | RPT3 non-catalytic | 19S (RP base) | 1.08 | 2.17 | 1.38 | 2.13 |
| XIcs AT5G10564 | TOP2 Met | Pl | 1.05 | 2.26 | 1.44 | 2.03 |
| XIcs AT1G21720 | PBC1 Thr | 20S (CP β) | 1.33 | 1.96 | 1.41 | 2.01 |
| XIcs AT1G56450 | PBG1 Thr | 20S (CP β) | 1.18 | 1.86 | 1.21 | 1.78 |
| XIcs AT5G42790 | PAF1 Thr | 20S (CP α) | 1.41 | 1.80 | 1.14 | 1.83 |
| XIcs AT4G01610 | CATIHB3 Cys | E,V | 1.13 | 1.84 | 0.85 | 1.79 |
| XIcs AT1G47128 | RD21A Cys | E,V | 0.91 | 1.46 | 0.74 | 1.57 |
| SC AT1G58350 | PAE1 Thr | 20S (CP β) | 1.75 | 1.69 | 1.50 | 1.67 |
| SC AT5G45890 | SAG12 Cys | V | 1.50 (+) | 22.50 (+) | 1.00 (+) | 24.50 (+) |
| Class 4 | XIcs AT5G51070 | CLPD Ser | Pl | 1.49 (+) | 2.37 (+) | 1.13 | 1.40 (+) |
| XIcs AT3G13225 | D01 Asp | C | 1.53 | 1.61 | 1.21 | 1.21 |
| XIcs AT1G79340 | AMC4 Cys | PM,C | 1.41 | 1.51 | 1.28 | 1.36 |
| XIcs AT1G50080 | _ Ser | C | 1.30 | 1.34 | 1.13 | 1.08 |
| Class 5 | XIcs AT3G51260 | PAD1 Thr | 20S (CP α) | 1.58 | 2.24 | 1.73 | 1.79 |
| XIcs AT5G23540 | RPN11 non-catalytic | 19S (RP lid) | 1.10 | 1.34 | 1.10 | 1.26 |
| XIcs AT4G33860 | RPN10 non-catalytic | 19S (RP lid) | 1.19 | 1.31 | 1.28 | 1.28 |
| XIcs AT1G51710 | UBP6 Cys | C | 1.13 | 1.22 | 1.06 | 1.05 |
| SC AT5G66140 | PAD2 Thr | 20S (CP α) | 1.25 | 5.00 | 2.33 | 1.67 |
| SC AT4G30910 | LAP3 Met | Pl | 0.89 | 1.35 | 1.20 | 1.45 |
| XIcs AT4G39090 | RD19A Cys | V,N | 1.15 | 1.74 | 0.96 | 1.56 |
| XIcs AT5G43060 | RD21B Cys | E,V | 0.99 (+) | 1.49 (+) | 0.59 | 1.36 |
| SC AT5G10760 | AED1 Asp | E | 1.80 (+) | 3.33 (+) | 0.67 | 1.50 (+) |
| Class 6 | SC AT1G16470 | PAB1 Thr | 20S (CP α) | 1.50 | 1.40 | 2.22 | 1.35 |
| SC AT1G79210 | PAB2 Thr | 20S (CP α) | 1.55 | 1.11 | 2.00 | 1.21 |
| XIcs AT2G14260 | PIP Ser | C,Pl | 1.18 | 1.23 | 1.48 | 1.24 |
| Class 7 | XIcs AT5G23140 | CLPP2 Ser | M | 0.93 | 2.42 | 0.86 | 2.77 |
| XIcs AT4G16190 | RD19C Cys | E,V | 1.05 | 1.46 | 1.18 | 2.08 |
| XIcs AT5G26860 | LON1 Ser | M | 1.21 | 1.31 | 1.02 | 1.40 |
| SC AT3G34290 | PAE2 Thr | 20S (CP α) | 1.40 | 1.33 | 1.42 | 1.63 |
| Class 8 | XIcs AT3G20630 | UBP14 Cys | C | 1.43 | 1.40 | 1.42 | 1.31 |
| Class 9 | XIIcs AT2G341790 | PXM16 Met | Pe | 1.54 | 1.29 | 1.43 | 1.11 |
| Class 10 | XIIcs AT4G36760 | APP1 Met | PM,C | 1.51 | 1.72 | 1.27 | 1.62 |
| Class 11 | XIIcs AT5G36210 | _ Ser | Pl | 1.36 | 1.24 | 1.57 | 1.31 |

Plants were grown under high- (HN) or low- (LN) nitrate conditions. (1) XIcs or Spectral counting (SC) methods. (2) Protein names according to UniProt and TAIR. (3) Catalytic classes according to MEROPS: asp, aspartic proteases; cys, cysteine proteases; met, metallo-proteases; ser, serine proteases; thr, threonine proteases; non-catalytic, non-catalytic proteasome regulatory sub-units. (4) Predicted subcellular localizations according to SUBA3 (SUBAcon), Bio-Analytic Ressource for Plant Biology (Cell eFP Viewer) and Marshall et al. (2015). 20S (CP α), proteasome 20S core protease α; 20S (CP β), proteasome 20S core protease β; 19S (RP base), proteasome 19S regulatory particle base; 19S (RP lid), proteasome 19S regulatory particle lid; C, cytosol; ER, endoplasmic reticulum; E, extracellular; M, mitochondrion; Pe, peroxisome; PM, plasma membrane; Pl, plastid; V, vacuole. (5) Fold-change ratios were calculated by dividing protein abundance in atg5 and atg5/sid2 by protein abundance in Col and in sid2, respectively. Entries in bold represent significantly increased accumulations, all other entries are non-significant changes (n = 3; P<0.05; ANOVA and Tukey's post-hoc test for multiple comparisons). (+) significant increase in gene expression (Log2 fold-change of mRNA levels >1.2 with P-value <10-5, rank product and FDR estimation).
grouped together four proteases over-accumulated in \textit{atg5/sid2} under LN. Two of them are essential for mitochondria maintenance (LON1 and CLLP2), whilst the others are a proteasome subunit (PAE2) and a cysteine protease RD19C (RESPONSIVE TO DEHYDRATION 19C). Classes 8-up, 9-up, 10-up, and 11-up included UBP14 (UBIQUITIN-SPECIFIC PROTEASE 14; accumulated in \textit{atg5} and \textit{atg5/sid2} under HN only), PXM16 (metallo-protease of the M16 family; only accumulated in \textit{atg5} under HN), APPI (accumulated in all conditions and \textit{atg} genetic backgrounds except in \textit{atg5/sid2} under HN), and the unknown serine protease (At5g36210; accumulated compared to the controls in \textit{atg5} and \textit{atg5/sid2} under HN, and in \textit{atg5/sid2} under LN). The ALEURAIN-LIKE PROTEASE, AALP (AT5G60360), was found by the shotgun LC-MS/MS proteome analysis to be slightly, but not significantly, increased in the \textit{atg} lines under LN (see Supplementary Fig. S3).

The proteases that had decreased accumulation were also classified. The two FTSH1 and FTSH5 (FILAMENTATION TEMPERATURE SENSITIVE H 1 and 5) chloroplast proteases and an unknown vacuole serine protease (At4g36195) were significantly less abundant in both \textit{atg5} and \textit{atg5/sid2}, and under both LN and HN (Class1-down; Table 2). Class2-down contained two extracellular-predicted proteases [AED3

| Class | XICs | Accession (TAIR10) | Name | Catalytic class | Predicted subcellular localization | Fold-change | \textit{atg5} | \textit{atg5/sid2} |
|-------|------|-------------------|------|---------------|-----------------------------------|-------------|----------|----------------|
| Class 1 | XICs | AT1G50250 | FTSH1 | met | Pl | 0.76 | 0.49 | 0.67 | 0.42 |
| Class 2 | XICs | AT4G42270 | FTSH5 | met | Pl | 0.71 | 0.51 | 0.67 | 0.48 |
| Class 3 | XICs | AT1G09750 | AED3 | asp | E,Pl | 0.66 | 0.61 | 0.77 | 0.52 |
| Class 4 | XICs | AT2G47940 | DEGP2 | Ser | Pl | 0.69 | 0.52 | 0.81 | 0.54 |
| Class 5 | XICs | AT5G08260 | SCPL25 | ser | E | 0.60 | 0.46 | 0.70 | 0.44 |
| Class 6 | XICs | AT2G39850 | SBT4.1 | Ser | E | 0.60 | 0.48 | 0.78 | 0.59 |
| Class 7 | XICs | AT1G52510 | AED3 | asp | E,Pl | 0.69 | 0.52 | 0.81 | 0.54 |
| Class 8 | XICs | AT2G47940 | FTSH5 | met | Pl | 0.71 | 0.56 | 0.81 | 0.58 |
| Class 9 | XICs | AT3G35780 | SCPL26 | Ser | E | 0.81 | 0.58 | 0.74 | 0.46 |
| Class 10 | XICs | AT5G39830 | DEGP8 | Ser | Pl | 0.81 | 0.57 | 0.75 | 0.67 |
| Class 11 | XICs | AT3G27925 | DEGP1 | Ser | Pl | 0.76 | 0.68 | 0.77 | 0.68 |
| Class 12 | XICs | AT3G14067 | SASP | Ser | E | 0.78 | 0.73 | 0.88 | 0.74 |
| Class 13 | XICs | AT3G19170 | PREP1 | met | Pl | 0.81 | 0.74 | 0.84 | 0.74 |
| Class 14 | XICs | AT5G42390 | SPP | met | Pl | 0.81 | 0.38 | 0.75 | 0.43 |
| Class 15 | XICs | AT3G24590 | PLSP1 | Ser | Pl | 0.69 | 0.36 | 0.75 | 0.50 |
| Class 16 | XICs | AT1G13270 | MAP1C | met | Pl | 0.70 | 0.56 | 0.71 | 0.48 |
| Class 17 | XICs | AT5G05740 | EGY2 | met | Pl | 0.78 | 0.55 | 0.85 | 0.56 |
| Class 18 | XICs | AT2G30950 | FTSH2 | met | E | 0.75 | 0.57 | 0.68 | 0.54 |
| Class 19 | XICs | AT4G18370 | DEGP5 | Ser | Pl | 0.66 | 0.54 | 0.83 | 0.65 |
| Class 20 | XICs | AT2G35250 | DEGP2 | Ser | Pl | 0.82 | 0.65 | 0.77 | 0.61 |
| Class 21 | XICs | AT5G42240 | SCPL42 | Ser | E | 0.86 | 0.66 | 0.87 | 0.62 |
| Class 22 | XICs | AT1G06430 | FTSH8 | met | Pl | 0.77 | 0.71 | 0.74 | 0.58 |
| Class 23 | XICs | AT5G23210 | SCPL34 | Ser | E | 0.86 | 0.65 | 0.87 | 0.68 |
| Class 24 | XICs | AT3G06350 | _ | met | Pl | 0.83 | 0.66 | 0.88 | 0.69 |
| Class 25 | XICs | AT5G66760 | _ | Ser | Pl,V | 0.86 | 0.70 | 0.93 | 0.68 |
| Class 26 | XICs | AT1G67700 | HHL1 | met | Pl | 0.76 | 0.39 | 0.74 | 0.53 |
| Class 27 | XICs | AT3G61820 | _ | Asp | E | 0.84 | 0.61 | 0.72 | 0.65 |
| Class 28 | XICs | AT1G03100 | APPI2 | Asp | E,PM | 0.86 | 0.64 | 0.73 | 0.73 |
| Class 29 | XICs | AT4G34980 | SLP2 | Ser | Pl | 0.81 | 0.71 | 0.87 | 1.16 |
| Class 30 | XICs | AT2G05920 | SBT1.8 | Ser | E,PM | 0.82 | 0.74 | 0.71 | 0.77 |
| Class 31 | XICs | AT3G18490 | ASPG1 | Ser | Pl | 0.93 | 0.81 | 0.85 | 0.60 |
| Class 32 | XICs | AT2G33530 | SCPL46 | Ser | Pl | 1.22 | 0.97 | 0.89 | 0.63 |
| Class 33 | XICs | AT4G21650 | SBT3.13 | Ser | Pl | 0.71 | 0.55 | 0.69 | 0.65 |
| Class 34 | XICs | AT3G08260 | SCPL35 | Ser | E | 0.90 | 0.83 | 0.66 | 0.65 |
| Class 35 | XICs | AT1G09130 | CLPR3 | Ser | Pl | 0.78 | 1.00 | 0.41 | 0.91 |

Plants were grown under high- (HN) or low- (LN) nitrate conditions. (1) Method for proteome analysis was XICs. (2) Protein names according to UniProt and TAIR. (3) Catalytic classes according to MEROPS: asp, aspartic proteases; met, metallo-proteases; ser, serine proteases. (4) Predicted subcellular localizations according to SUBA3 (SUBAcon) and Bio-Analytic Ressource for Plant Biology (Cell eFP Viewer). E, extracellular; PM, plasma membrane; PI, plastid; V, vacuole. (5) Fold-change ratios were calculated by dividing protein abundance in \textit{atg5} and in \textit{atg5/sid2} by protein abundance in Col and in \textit{sid2}, respectively. Entries in bold represent significantly decreased accumulations, all other entries are non-significant changes ($n = 3$; $P < 0.05$; ANOVA and Tukey’s post-hoc test for multiple comparisons). (–) significant decrease in gene expression (Log$_2$ fold-change of mRNA levels $< -1.2$ with $P$-value $< 10^{-5}$, rank product and FDR estimation).
(APOLYASTIC, EDS1-DEPENDENT 3) and the product of AT3G54400. Class3-down (down in atg5 under both LN and HN, and in atg5/sid2 under LN) principally included several serine proteases, almost all predicted in the extracellular space (such as SASP, SENESCECE-ASSOCIATED SUBTILISIN PROTEASE), or in the plastids (such as the DEGRADATION OF PERIPLASMIC PROTEINS DEGP1, DEGP2, and DEGP8 proteases, and the PREP1 zinc metallo-protease). Class4-down clustered together proteases that were decreased in atg5 and atg5/sid2 only under LN. They were almost all plastid- (FTSH2, FTSH8, DEGP5) or extracellular-predicted, and they mostly belong to the serine and metallo-protease families. Class5-down grouped together proteases repressed in atg5 under LN, and mainly included extracellular proteases, but also contained the HHLL1 (HYPERSENSITIVE TO HIGH LIGHT 1) metallo-protease that is involved in the protection of photosystem II (Jin et al., 2014). Class6-down contained proteases differentially repressed in the atg5/sid2 mutant under LN. They are almost all extracellular-predicted and serine proteases except for ASPG1 (ASPARTIC PROTEASE IN GUARD CELL 1), which is extracellular-predicted, but also localizes in the endoplasmic reticulum in the guard cells (Yao et al., 2012). Class7-down and Class8-down both contained only one member: the extracellular SCPL35 (decreased accumulation in atg5/sid2 under LN and HN) and the chloroplast CLPR3 (decreased accumulation in atg5/sid2 under HN), respectively.

From these results, it clearly appears that the protease-related proteins accumulated in atg5 and/or atg5/sid2 mostly belonged to the proteasome machinery and to the cytosolic or vacuole cysteine protease families. The proteases with decreased accumulation were mainly ones with chloroplast- or extracellular-predicted localizations and belonged mainly to the serine- and metallo-protease families. It should be noted that the decrease of chloroplast-predicted proteases in atg5 was independent of SA (observed in both the Col and
sid2 backgrounds) and independent of N (observed under 
HN as well as under LN). It is thus likely that the decrease 
in these chloroplast-predicted proteases was not correlated to 
the slight senescence phenotype observed in atg5 under low- 
nitrate conditions (Fig. 1b), but constitutes a more robust 
phenotype that was shared by the two autophagy-defective 
lines studied, independently of the nitrate conditions.

In order to determine whether changes in protease con- 
tents were linked to a modification of the transcription of 
their genes or to modifications in their protein turnovers, 
gene expression levels were evaluated using the transcrip- 
tomic data obtained by Masclaux-Daubresse et al. (2014), 
(Supplementary Dataset S1). Only the CLPD, RD21B, 
AED1, and SAG12 genes were up-regulated in atg5 and/or 
atg5/sid2 (Table 1), and only SBT3.13 was repressed in atg5 
under low nitrate (Table 2). Thus, the changes in the protease 
protein contents reported in Tables 1 and 2 were more likely 
due to modifications in their turnover rates.

Proteasome and papain-like cysteine protease 
activities are higher in the atg5 and atg5/sid2 lines, but 
not serine protease activity

It is well known that protease activities can be regulated at sev- 
eral post-transcriptional levels. In addition, it has been shown 
in the case of the proteasome, for example, that autophagy 
could play a role in proteaphagy (Marshall et al., 2015). Thus, 
in order to determine whether the accumulation or depletion 
of proteases detected in the atg5 mutant lines caused modifi- 
cations in protease activities, we explored changes in specific 
protease activities.

The degradation of the Rubisco large sub-unit (RBCL) at 
acidic pH was monitored using specific protease inhibitors 
according to the procedure described by Poret et al. (2016). 
We observed that RBCL degradation was higher in the two 
atg5 lines relative to controls (see Supplementary Fig. S4a) 
and that degradation was inhibited when the E-64 cysteine-
protease inhibitor or the aprotinin serine protease inhibitor 
were added before incubation of the extract (Fig. S4b).

ABPP was performed in order to detect changes in the 
contents of active proteasome sub-units, active cysteine pro-

tases, and active serine proteases (Morimoto and van der 
Hoorn, 2016). For the proteasome, the fluorescent probe 
MVB072 was added to the protein samples in order to deri-

tivatize the active proteasome protease sub-units. Fluorescent 
derivatized sub-units were then detected after SDS-PAGE 
separation (Fig. 4). MVB072 competition assays were carried 
out to estimate the probe’s specificity toward the proteasome 
sub-units (see Supplementary Fig. S5). Significant increases 
in the proteasome-active β1, β2, and β5 sub-units (encoded 
by PBA1, PBB1/PBB2 and PBE1/PBE2 genes respectively) 
were detected in atg5 under HN, and in both atg5 and atg5/
sid2 under LN. By contrast with PBA1 (proteasome-active 
β1 subunit), no PBB1 or PBE2 subunits were identified in the 
shotgun analyses. These increases suggested that the prote-

asome activity was increased in autophagy-defective lines and 
especially under LN.

In order to detect active papain-like cysteine proteases 
(PLCPs), DCG-04 was added to the protein samples before 
SDS-PAGE separation. The biotin-tag that linked to the 
active PLCP was detected on membranes by western blot 
analysis using streptavidin-HRP chemiluminescence (Fig. 5). 
Five bands were detected on the membranes and their chemi-

luminescence signal was clearly higher in atg5 and atg5/sid2 
relative to Col and sid2, respectively, and especially under LN 
(Fig. 5a). The controls performed to demonstrate the specific- 
ity of the synthetized DCG-04 toward cysteine proteases are 
presented in Supplementary Fig. S6.

The active serine proteases were detected using desthio-

biotin-FP, and at both acidic and neutral pH as serine
proteases are localized in both vacuoles and chloroplasts (see Supplementary Figs. S7 and S8). Surprisingly, no differences between the atg5 lines and control lines were detected. This suggests that the global serine protease activity was not different in the autophagy-defective lines compared to the control lines (Supplementary Fig. S8). Inconsistent with the data obtained using the aprotinine serine protease inhibitor on RBCL degradation at pH 5.5, this result suggests that the serine protease activities monitored using desthiobiotin-FP were not the same as those that degraded RBCL in our previous assay.

Identification of active papain-like cysteine proteases in autophagy mutants.

Given that LC-MS/MS analyses showed that cysteine protease proteins accumulated in the autophagy mutants, and that DCG-04 revealed several active PLCP bands on membranes, a pull-down of active PLCPs was carried out using DCG-04. Co-precipitated PLCPs were separated on SDS-PAGE gels and streptavidin-HRP reactive bands were analysed using shotgun LC-MS/MS. This assay was only carried out on extracts from plants grown under LN conditions, as no differences had been detected on gels between genotypes when grown under HN (Fig. 5). In this experiment, spectral counting was used to determine the relative amounts of active PLCPs in the different genetic backgrounds (see Supplementary Table S1). The results showed that all the over-accumulated PLCPs previously identified in the autophagy-defective lines were active. The largest differences detected between the atg5 and control lines after DCG-04 pull-down were for RD21A and SAG12 (Fig. 6b). Active CATHB3,
AALP, RD21B, RD19A, and RD19C were also more abundant in \( \text{atg5} \) and \( \text{atg5/sid2} \) (Fig. 6c) Additional cysteine proteases were found that had not been identified by the shotgun LC-MS/MS whole-proteome analysis. They were CEP1 (CYSTEINE ENDOPEPTIDASE 1; At5g50260), which is involved in tapetum cell death and pathogen defence (Zhang et al., 2014), CATHB2 (CATHEPSIN-LIKE B2 CASPASE-LIKE; At1g02305), which is senescence-induced (Ge et al., 2016), and XCP1 (XYLEM CYSTEINE PEPTIDASE 1; At4g35350), which is a xylem cysteine peptidase (Avci et al., 2008). It should be noted that CEP1 was over-expressed at the transcriptional level in \( \text{atg5} \) and \( \text{atg5/sid2} \), and under both \( \text{HN} \) and \( \text{LN} \).

**Western blot identification of mature and immature forms of papain-like cysteine proteases**

Antibodies raised against RD21A, SAG12, and CATHB3 were used in order to verify the accumulation of their processed forms and to monitor their immature forms in the \( \text{atg5} \) lines under \( \text{LN} \) and also under \( \text{HN} \) (Fig. 7). The RD21A intermediate form (i) was detected in both \( \text{atg5} \) and control lines, and under both \( \text{LN} \) and \( \text{HN} \). Its level was very high in all the lines, although it was higher in the \( \text{atg5} \) lines than in controls. The mature forms (m1 and m2; Fig. 7) were much less abundant than the intermediate forms, but still more abundant in the \( \text{atg5} \) lines than in controls. The m2 form was notably higher in the \( \text{atg5} \) lines under \( \text{LN} \) than under \( \text{HN} \), and in \( \text{atg5/sid2} \) than in \( \text{atg5} \) under \( \text{LN} \). For SAG12, the immature signal was weak, but higher in the \( \text{atg5} \) lines grown under \( \text{LN} \). The mature form (m) was only observed in the \( \text{atg5} \) lines under low \( \text{N} \) and was much stronger than the immature form (i), in contrast with RD21A. All the CATHB3 mature and immature forms were more abundant in the \( \text{atg5} \) lines relative to the control lines, Col and \( \text{sid2} \). The intermediate form with a processed C-terminal domain (\( \Delta \text{C} \)) and the mature form (M) of CATHB3 were especially higher in the \( \text{atg5} \) lines than in the controls under low-nitrate conditions. Therefore, western blots confirmed that the active forms of RD21A, SAG12, and CATHB3 were more abundant in the autophagy-defective lines.

**Inhibitor proteins of cysteine proteases are differentially accumulated in autophagy-defective lines**

Several protease inhibitors are small proteins that can form stable complexes with target proteases and block or prevent access to the active site. Among the long list of protease inhibitors available in the MEROPS database, three were found to be differentially accumulated in autophagy-defective
The aim of this study was to identify protease activities up-regulated in autophagy mutants in order to further determine their roles in nitrogen remobilization for seed filling (Guiboileau et al., 2012). Shotgun LC-MS/MS and activity-based protein profiling (ABPP; Morimoto and van der Hoorn, 2016) analyses showed that, while protease genes were not transcriptionally induced or repressed in the autophagy mutants (atg5 and atg5/sid2) relative to their controls (Col and sid2, respectively), many proteases were differentially abundant in the atg mutants (Fig. 8).

The proteases depleted in the atg mutants were mainly chloroplast- and extracellular-predicted proteases. Several chloroplast FTSH and DEG proteases were less abundant in the atg5 lines (Kato and Sakamoto, 2010; van Wijk, 2015). FTSH-1, -2, -5, and -8 are the four major isomers of the chloroplast FTSH complexes involved in thylakoid maintenance (Yu et al., 2004). Their decrease in the atg lines, especially under LN, suggested that thylakoid maintenance was affected. The DEGs ATP-independent serine-type proteases may be involved in the degradation of thylakoid luminal proteins and of PSI (Chassin et al., 2002) and DEGP2 may be responsible for the endoproteolytic cleavage of the protein D1 (Haußühl et al., 2001). Hence, together with the decrease of FTSH proteins, the decrease of DEGP-1, -2, -5, and -8 protein abundance suggests that thylakoid and photosystem maintenance are affected in atg lines. The number of chloroplast proteases with decreased accumulation in the autophagy-defective lines was slightly higher under nitrate starvation, but was not dependent on the leaf-senescence phenotype or on SA synthesis. The lower abundance of chloroplast proteases in the autophagy-defective lines suggests that chloroplasts could be less abundant or less active in the atg5 lines than in the wild-type, thus explaining the lower carbon, starch, and sugar contents measured in atg5, atg9, and atg18a-RNAi by Guiboileau et al. (2013) and Masclaux-Daubresse et al. (2014).

In addition to chloroplast proteases, many extracellular-predicted subtilisin-like proteases (SBTs) and extracellular-predicted serine carboxypeptidase-like proteases (SCPLs) were decreased in the autophagy-defective lines. The SBTs have mainly been described in plant–pathogen or pest-defence responses (Figueiredo et al., 2014). The SCPLs are more likely involved in secondary metabolism and could also have a role in plant defence (Liu et al., 2008; Mugford and Osbourn, 2010). The current understanding of the function of SBTs remains very limited, although a role for some of them in plant immune priming has been proposed (Figueiredo et al., 2014). It is difficult to explain why the atg5 and atg5/sid2 lines had reduced amounts of extracellular SBTs and SCPLs. Dysfunction in exocytosis in the atg mutants could be one explanation, although a relationship between exocytosis and autophagy remains to be clearly demonstrated in plants (Wang et al., 2010; Lin et al., 2015; Münz, 2017). Nevertheless, the lower extracellular content of SBTs in atg5 might explain the higher susceptibility of the autophagy mutants to necrotrophic pathogens, as reported by several studies (Lai et al., 2011; Lenz et al., 2011). Martinez et al. (2015) recently reported that the extracellular senescence-induced SASP subtilisin protease was involved in branching; the authors hypothesized that SASP could be
involved in the processing of extracellular apoplastic signals repressing inflorescence branching and delaying leaf senescence. Although we found decreased accumulation of SASP in the atg5 lines, no branching phenotype could be observed, but senescence was possibly enhanced.

Among the proteases with increased accumulation in the autophagy-defective lines, it was noticeable that many were proteasome sub-units and that several proteins were related to the ubiquitin proteasome system (UPS), such as LAP1, TOP2, TPP2, UCH3, and UBP14. The UPS and autophagy are two major pathways that degrade most of the cellular proteins of eukaryotic cells. It is considered that while the proteasome is responsible for the turnover of short-lived proteins, autophagy is more specifically dedicated to the degradation of long-lived or aggregated proteins (Lilienbaum, 2013, and references therein). It appears that the UPS and autophagy are tightly controlled and co-ordinated. Several studies have suggested that autophagy may act as a back-up system, thereby assisting degradation in case the UPS is overloaded (Nedelsky et al., 2008). It seems that most of the mis-folded soluble proteins that are preferentially degraded by the UPS in cases of endoplasmic reticulum (ER) stress can be degraded through the autophagy pathway if the proteasome capacity is exceeded (Johnston et al., 1998). Several studies have also suggested that the proteasome itself could be degraded by autophagy (Waite et al., 2016). In plants, the accumulation of several proteasome sub-units in autophagy-defective mutants has been reported. Marshall et al., (2015) showed that RPN10 could act as a selective autophagy receptor that targets ubiquitinated and inactive 26S proteasomes and binds to ATG8 for autophagy-mediated degradation. We also found that several proteasome sub-units were more abundant in atg5, especially under LN, including RPN10 (Table 1). This suggests that proteophagy was increased under LN and was compromised in atg5. It thus seems that low-N conditions that trigger N remobilization (Masclaux-Daubresse and Chardon, 2011) can induce selective proteaphagy (Waite et al., 2016; Zientara-Rytter and Sirko, 2016).

With regards to the proteasome, our ABPP assay showed that active β1, β2, and β5 catalytic subunits of the proteasome were more abundant in atg5 under LN and HN and also in the atg5/sid2 defective line under LN. This suggested that the activity of the proteasome was globally higher in the autophagy-defective lines. It has been shown that ER stress is increased in plant autophagy mutants (Munch et al., 2014; Yang et al., 2016). A role for the proteasome in the endoplasmic reticulum-associated degradation of mis-folded proteins under ER stress has been demonstrated in mammals (Lee et al., 2001). We can therefore suspect that an increase in proteasome activity in atg5 and atg5/sid2 could be linked to ER stress.

### Table 3. List of protease inhibitors differentially accumulated in atg5 versus Col, and atg5/sid2 versus sid2

| Accession (TAIR10) | Name(1) | Description (2) | Predicted subcellular localization(3) | Fold-change(4) |
|--------------------|---------|-----------------|--------------------------------------|---------------|
| AT1G21750          | PDIL1-1/PDI5 | protein disulfide isomerase | ER | 1.77 2.45 1.39 1.95 |
| AT1G47710          | SERPIN-1 | ser protease inhibitor | C | 1.23 1.23 1.11 1.13 |
| AT4G16500          | CYS4 | cys protease inhibitor | E | 0.92 0.77 0.77 0.55 |

Plants were grown under high- (HN) or low- (LN) nitrate conditions. (1) Protein names according to UniProt and TAIR. (2) Description (TAIR10). (3) Predicted subcellular localizations according to SUBA3 (SUBAcon) and Bio-Analytic Resources for Plant Biology (Cell eFP Viewer). C, cytosol; ER, endoplasmic reticulum; E, extracellular. (4) Fold-change ratios were calculated by dividing protein abundance in atg5 and atg5/sid2 by protein abundance in Col and sid2, respectively. Entries in bold cells represent significantly increased or decreased accumulations, all other entries are non-significant changes (n = 3; P<0.05; ANOVA and Tukey’s post-hoc test for multiple comparisons).
In addition to the accumulation of UPS proteins, increases of several cysteine, serine, and metallo-proteases were observed in the atg5 and atg5/sid2 lines. Activity measurements, performed using protease inhibitors, indicated that cysteine protease activities were significantly different between the atg5 and atg5/sid2 lines and the Col and sid2 controls. As they are suspected to play a fundamental role in N remobilization during senescence and in response to nitrate shortage, we then focused on the papain-like cysteine proteases (PLCPs; Avice and Etienne, 2014; Diaz-Mendoza et al., 2016; Pružinská et al., 2017). Pull-down using DCG-04 showed that active PLCPs were more abundant in the atg5 and atg5/sid2 lines (Table 1). Active SAG12, RD21A, CATHB3, and AALP were strongly enriched in atg5 and atg5/sid2, as well as RD19A, RD19C, CEP1, CATHB2, and XCP1, although to a lower extent. With the exception of SAG12 and CEP1, PLCP genes were not differentially expressed in atg5 versus Col or atg5/sid2 versus sid2. This absence of a significant change at the transcriptional level suggests that the accumulation of PLCPs in atg5 and/or atg5/sid2 was mainly due to higher translation or post-translational maintenance. Most of the PLCPs identified were predicted in the vacuole, except for RD21s that have been also found in ER-bodies (Hayashi et al., 2001), SAG12 that was detected in senescence-associated vacuoles (Otegui et al., 2005), and CEP1 that seems only to be located in the ER (Zhang et al., 2014). Many of the PLCPs, such as RD21s, RD19s, and CATHB3, transit through the ER before being delivered into the vacuole lumen where they play their protease functions. Whether autophagy could play a role in the trafficking of these proteases from the ER to the vacuole, as was suggested for seed-storage proteins (Michaeli et al., 2014), is unknown. The western blots performed using the RD21A, CATHB3, and SAG12 antibodies revealed that both mature and immature forms were accumulated the atg5 and atg5/sid2 lines, thus suggesting that there was no defect in protease maturation or trafficking in the autophagy mutants.

All the PLCPs identified in this study are known proteases. They have been described in several reports but their regulation, maturation, and physiological roles remain largely enigmatic (van der Hoorn, 2013; Rogers, 2013; Iglesias-Fernández et al., 2014; Zhang et al., 2014; Pathirana et al., 2017). Several are known to be transcriptionally induced during leaf senescence, such as SAG12, CATHB3, CATHB2, AALP, CEP1, RD21A, RD21B, RD19A, and RD19C (Supplementary Table S2; Noh and Amasino, 1999; Havé et al., 2017; Pružinská et al., 2017). However, as none of them except SAG12 and CEP1 were transcriptionally induced in the autophagy-defective lines relative to the controls, the possibility that these PLCPs were more abundant in atg5 and atg5/sid2 due to early senescence is weak. All these PLCPs were found to be more abundant in both atg5 and atg5/sid2 under LN but not under HN, and hence it is highly possible that they are involved in N remobilisation processes (Masclaux-Daubresse and Chardon, 2011), and are induced under low N and especially when autophagy is impaired. These proteases are thus good candidates as enzymes involved in the N-recycling pathways that lead to the remobilization of organic nitrogen forms from the source leaves to the sink leaves and to the seeds (Desclos et al., 2009). Their potential roles as back-up proteolytic systems to compensate for weaknesses in autophagy, or as the last step of an autophagy process and cargo degradation inside the vacuole, need verification. Pružinská et al. (2017) have recently shown that only knockout lines lacking AALP present delayed leaf senescence under dark stress, in contrast with the rd21a, rd21b, sag12, and cathb3 mutants. It therefore seems that AALP may be the best candidate for playing a role in N remobilization, together with, or in parallel to, autophagy.

It is well known that autophagy mutants exhibit spontaneous regions of cell death on their leaves with ageing (Masclaux-Daubresse et al., 2014). The role of the PLCPs identified here in this phenotype of the autophagy mutants can be questioned (Lampl et al., 2013; Iglesias-Fernández et al., 2014; Cai et al., 2018; Zhang et al., 2014). Indeed, several reports suggest that AMC4, CATHB3, PBA1, and RD21s might play a role in the control of cell death (Watanabe and Lam, 2011; Cai et al., 2018), either as cell death-promoting or longevity factors. Interestingly, we found that AMC4 accumulated in atg5 but not in atg5/sid2 whatever the nitrate conditions, while RD21A, CATHB3, and SAG12 accumulated in both atg5 and atg5/sid2 but only under LN. These discrepancies in accumulation patterns possibly distinguish the different functions of these proteases. Proteases promoting death should be independent of nitrate but SA-dependent, such as AMC4, while the N-remobilization proteases should be SA-independent but nitrate-dependent, such as RD21A, CATHB3, and SAG12. Hypotheses such as these need further investigations with mutants in order to be confirmed.

Changes in protein inhibitors of proteases were also identified. Increases in PDIL1/PD15 and in Serpin-1 proteins suggested additional controls exerted on the RD21 proteases in the atg5 and atg5/sid2 lines to moderate their activity (Lampl et al., 2013; Rustgi et al., 2017). The decrease of CYS4 suggests that its unknown cysteine protease partners are repressed in the autophagy-defective lines (Je et al., 2014).

In conclusion, we identified several proteases that are differentially accumulated in autophagy-defective lines compared to their controls, and that could explain the phenotype of autophagy mutants such as the spontaneous regions of cell death on the leaves and the lower carbon and sugar contents due to reduced chloroplast maintenance. Many of the PLCPs identified could play a compensatory role for protein degradation and nutrient recycling, especially under low nitrate nutrition. Further investigations and biochemical studies are required using protease and autophagy mutants to clarify their contributions.

Supplementary data

Supplementary data are available at JXB online.

Methods S1–S3. Phenotyping display and growth conditions; details of the shotgun proteomics analysis; and details of the shotgun LC-MS/MS analysis of pulled-down PLCPs.

Fig. S1. α-mRD21A and cathepsin B3-N Term-1610N antibodies are specific for Arabidopsis RD21A and cathepsin B3, respectively.
Fig. S2. Salicylic acid concentrations in atg5-1sid2 and atg5-2/NahG grown under low-nitrate conditions.
Fig. S3. ALEU protease amounts tend to increase in autophagy-defective mutants.
Fig. S4: Rubisco degradation in atg defective lines and controls.
Fig. S5. MVBO72 competition assay to estimate the probe’s specificity toward proteasome sub-units.
Fig. S6. DCG-04 competition assay to estimate the probe’s specificity toward PLCPs.
Fig. S7. Desthiobiotin-FP competition assay to estimate the probe’s specificity toward serine proteases.
Fig. S8. Serine protease activities are stable in autophagy mutants.

Table S1. Details of spectral counts for PLCP peptides in Col, sid2, atg5, and atg3/5/sid2.

Table S2.: Senescence up- and down-effects on the transcription of protease and protease-inhibitor genes.

Dataset S1. Transcriptomic data obtained by Masclaux-Daubresse et al. (2014).

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Author contributions

MH performed all the experiments with technical help from ED, GC, FS, and TB ran the LC-MS/MS analyses. MH and TB collected and analysed the LC-MS/MS data. BC-B synthesized the DCG-04 probe. PG provided the CATHB3 antibody and performed controls on cathb3 mutants to certify antibody specificities. ND and PR provided the anti-RD21A antibody and performed controls on the rd21a mutant and recombinant mRD21A protein. MZ and LR participated in the design of the biochemical experiments and in discussions. AL and J-CA designed and performed the Rubisco degradation assays. CM-D designed and supervised the research. MH and CM-D interpreted the results and wrote the manuscript.

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