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Research Article

Dynamic hydrolase labelling as a marker for seed quality in Arabidopsis seeds

Natanael Viñegra de la Torre[1], Farnusch Kaschani[2], Markus Kaiser[2], Renier A. L. van der Hoorn[3], Wim J. J. Soppe[1,*] and Johana C. Misas Villamil[1,4]

[1]Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany; [2]Chemische Biologie, ZMB, Fakultät für Biologie, Universität Duisburg-Essen, 45117 Essen, Germany; [3]The Plant Chemetics Laboratory, Department of Plant Sciences, University of Oxford, Oxford, U.K.; [4]The Terrestrial Microbiology Lab, Botanical Institute, University of Cologne, 50674 Cologne, Germany

Correspondence: Johana C. Misas Villamil (jmisas@uni-koeln.de)

Seed quality is affected by different constituents of the seed. In general, seed lots are considered to be of high quality when they exhibit fast and homogeneous germination. When seeds are stored, they undergo different degrees of damage that have detrimental effects on their quality. Therefore, accurate prediction of the seed quality and viability levels of a seed lot is of high importance in the seed-producing industry. Here, we describe the use of activity-based protein profiling of proteases to evaluate the quality of artificially and naturally aged seeds of *Arabidopsis thaliana*. Using this approach, we have identified two protease activities with opposite behaviours in aged seeds of Arabidopsis that correlate with the quality status of the seeds. We show that vacuolar processing enzymes (VPEs) become more active during the ageing process, in both artificial and natural ageing treatments. Secondly, we demonstrate that serine hydrolases are active at the beginning of our artificial ageing treatment, but their labelling decreases along with seed viability. We present a list of candidate hydrolases active during seed germination and propose that these protease activities can be used in combination with VPEs to develop novel markers of seed quality.

Introduction

Seed quality is widely understood in terms of a seed's nutritious value, a concept that provides a reference to the seed's content of lipids and proteins. Nevertheless, seed quality can also refer to a subset of seed traits which will determine its performance and agronomic value. Seed quality is determined by several factors, such as genetic purity, germination capacity and uniformity, vigour, storability and performance, under suboptimal conditions [1]. Generally speaking, high-quality seeds will germinate close to 100%, germinate quickly and at the same time, generate normal and healthy seedlings, show little variation of germination in response to external factors and have high storability [2]. It is a major challenge for the seed industry to fulfil these demands as well as to develop methods to evaluate and discriminate the quality level of seed lots. In this paper, we define seed quality more narrow as the germination percentage of a seed batch [3,4].

Seed deterioration during storage has detrimental effects on seed quality, as it involves damage to cell constituents as well as disruption of the cell machinery. The main contributors to this deterioration during storage are free radical-mediated lipid peroxidation, loss of integrity of nucleic acids, disruption of cellular membranes, enzyme inactivation and protein degradation [5–8]. Most of these damages originate from oxidative processes, which, in most cases, lead to the production of reactive oxygen species (ROS) that can act as signalling molecules [9] or, at higher concentrations, damage cellular components [10]. For example, dry seeds were shown to release volatiles associated with lipid peroxidation or alcoholic fermentation [11–13]. A particular case of chemical oxidation occurring in

*Present address: Rijk Zwaan, De Lier, 2678 ZG, Netherlands.

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dry seeds is the Maillard reaction [14] in which, through a non-enzymatic process, reducing sugars or aldehydes react with the amino groups present in proteins. This process results in glycosylated end-products whose accumulation negatively affects seed longevity [15].

Environmental conditions are of key importance for seed storage. Factors such as temperature and relative humidity (RH) have profound effects on seed longevity and on the rate of seed ageing [16–18]. The combination of low temperatures and reduced seed moisture content ensures that the seed's cytoplasm stays in the glassy state, minimising the deleterious impact of ageing [6]. Increasing temperature or moisture content of the seeds softens the cellular glass to a rubbery state or even back to the liquid state, reactivating metabolism and deteriorative processes. The modification of these two parameters is the theoretical basis for artificial ageing methods, which use controlled environments to replicate the natural ageing process in relatively short periods of time [19]. Based on this, the germination of seeds after artificial ageing is considered to be a vigour test and remains, to date, as the most reliable measurement to quickly assess seed quality [19]. The comparability between natural and artificial ageing of seeds has been a matter of study for some time and found to be similar in *Arabidopsis thaliana* and, to some extent, in rice [8,20].

Considering the crucial role of seeds in plant breeding and agriculture, the need of evaluating seed viability and vigour of any given commercial seed lot is apparent and fundamental for seed conservation and the seed industry, respectively. In particular, the development of methods to evaluate seed quality fast and reliably is of high interest. Activity-based protein profiling (ABPP) is a powerful biochemical approach that monitors the availability of functional sites correlated with the active functional status of proteins in an isolated proteome or in living cells [21]. This technique relies on the use of small, reporter-tagged probes that specifically react with the active site of an enzyme in a mechanism-dependent manner [21,22]. Probes are designed to bind covalently and irreversibly to certain enzyme subfamilies, allowing us to monitor specific proteins in their active state. The application of ABPP in plant research has allowed to observe the dynamic enzymatic activities taking place during pathogen infection [23], seed germination [24] or leaf senescence [25], providing clues to elucidate the underlying biochemical processes [26,27]. During the ageing process, seeds endure oxidative stresses, which damage cell constituents and progressively reduce the seed's capacity to germinate [28]. Among these cellular components, proteins are also affected by oxidative damage and rendered inactive by processes such as carbonylation [8,10]. Similarly, seed proteins can also undergo spontaneous age-related oxidative damage, such as the formation of abnormal amino acid residues which have detrimental effects on seed longevity [29–31]. Considering the specificity of ABPP probes for active targets and the above-mentioned effects of seed ageing, it is plausible that these probes and their target proteins could be used to monitor seed viability and quality.

Plant proteases are a wide group of enzymes involved in the degradation of non-functional proteins by cleaving specific peptide bonds. They also participate in responses to environmental and developmental cues, as well as immune responses [27,32]. In seeds, these enzymes are active during development and germination. During seed development, proteases participate in the accumulation of storage proteins, whereas in germination they contribute to the remobilisation of these resources [33,34].

In this study, we describe the use of ABPP as a tool to monitor the viability levels of artificially aged Arabidopsis seeds and provide insight into the molecular processes affected by ageing of seeds. We have identified enzymatic activities with opposite trends in aged seeds. First, we have shown that vacuolar processing enzymes (VPEs) become more active throughout the artificial ageing treatment, correlated with the loss of seed viability. Conversely, we have observed that ageing reduces the activity of serine hydrolases. Additionally, we have conducted a serine hydrolase pull-down assay in non-aged seeds and identified eighteen potential candidates as markers for seed viability.

Materials and methods

Plant material

In this work, *A. thaliana* accessions Columbia (Col-0) and Landsberg *erecta* (L*er*) were used. All the *vpe* mutants used were previously described [35].

Seed batches were obtained from plants cultivated on soil in a growth chamber with a 16-h-light/8-h-dark cycle (22/16°C). All germination assays presented in this work used seed batches obtained at the same time from the same environment. All germination experiments were conducted using independent biological replicates. A biological replicate consists of a batch of seeds harvested from a single mother plant.

Germination tests and accelerated ageing of seeds

For a germination test, 50–100 seeds were plated onto a filter paper soaked with demineralised water in Petri dishes. These plates were then incubated in a growth chamber under controlled conditions (16 h light /8 h darkness, at 25/20°C). After 7 days, radicle emergence was scored.

Seed viability was determined by quantifying the number of germinated seeds after different periods of accelerated ageing [19]. For this, aliquots of 100–200 seeds were placed on open PCR tube-stripes and incubated in hermetically sealed boxes containing a saturated solution of KCl at the bottom which, when placed at 37°C, generates an atmosphere of 85% RH within the container. Seeds were maintained at these conditions for the indicated periods, after which their final germination capacity was evaluated in a germination test.

Protein isolation and labelling

The first screening of the probes was conducted on proteins extracted directly in water, which were then immediately transferred to the corresponding buffer. This is the case for the labelled extracts shown in Supplementary Figures S1 and S2. In these experiments, the negative-labelling control was conducted on the water used for seed imbibition. After observing the differential labelling for two of the probes, all new protein extractions were performed directly on the buffer used for labelling, to improve the quality of the fluorescence signals, as described below.

For protein extraction, ~ 20 mg seeds of Arabidopsis were imbibed for 24 h, subsequently ground in liquid nitrogen to a fine powder and immediately suspended in 600 µl of the appropriate extraction buffer. For probe JOPD1, this buffer contained 67 mM sodium acetate at pH 5.5 including 10 mM dithiothreitol (DTT), whereas for FP probes, the buffer contained 67 mM Tris–HCl at pH 7.5 including 10 mM DTT. For probe MV151, the buffer used was either sodium acetate at pH 6.0 (when labelling PLCPs) or Tris–HCl at pH 7.5 (when labelling the 26 s proteasome), both including 10 mM DTT. Protein extracts were then centrifuged at least three times at 21 000×***g*** for 20 min, recovering the supernatant in a new microcentrifuge tube every time, until no oil traces or debris were visible.

For the labelling, 60 µl of protein extract was incubated with the probe for 2 h in the dark. The final concentrations of probe used for labelling were 1 µM JOPD1, 1 µM MV151 and 0.2 µM FP-rhodamine (FP-Rh). After incubation, the labelling reactions were stopped by adding 20 µl of 4× SDS–PAGE loading buffer. For negative-labelling controls, a pooled sample of protein extracts was first incubated for 30 min in the dark with a specific inhibitor of the enzymatic activity studied before adding the probe (Table 1).

These inhibitors were used at a final concentration that allowed them to out-compete the tested probe and therefore inhibit the labelling. An additional no-probe control (NPC) was prepared for each set of samples by combining a small fraction from each proteome. In this control, probe and inhibitor were not added and they were substituted by dimethyl sulfoxide (DMSO), which was the solvent for both probes and inhibitors.

Visualisation of labelled protein extracts and band quantification

Labelled protein samples were boiled for 5 min at 95°C prior to loading. These protein extracts were loaded in NuPAGE™ 4–12% Bis-Tris Protein Gels (ThermoFisher) and separated by electrophoresis [38] applying 120–130 V for 65–75 min. To preserve the fluorescent probes from degradation, the electrophoretic chamber was fully covered, so protein electrophoresis took place in the dark.

After separation, labelled proteins were visualised by in-gel fluorescence scanning using a Typhoon FLA 9000 scanner (GE Healthcare) with excitation and emission at 532 and 580 nm, respectively. After scanning,

Table 1. ABPP probes tested in this work
For each probe, its target, the pH at which the protein extract was isolated, the inhibitor and concentration used, and the reference is indicated.

Probe	Target	pH	Inhibitor	References
JOPD1	VPEs	5.5	YVAD-CMK (50 µM)	[23,24]
MV151	26 S proteasome	7.5	Epoxomicin (50 µM)	[36]
MV151	PLCPs	6.0	E64 (20 µM)	[36]
FP-Rh	Serine hydrolases	7.5	DiFP (100 µM)	[37]

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gels were stained with SYPRO® Ruby (ThermoFisher) for total protein assessment following the manufacturer's indications and scanned with excitation and emission at 450 and 610 nm, respectively. Quantification of the observed fluorescent bands was performed using the software ImageQuant TL 7.0 according to the manufacturer's indications (GE Healthcare). Briefly, bands of interest were fluorescently quantified in the gels presented in Figures 2 and 5. These measurements were then normalised against the total protein loaded stained with Sypro Ruby by measuring a band with similar labelling during the treatment. The relative intensities presented in Supplementary Figures S3 and S4 correspond to the ratio between these measurements.

Pull-down of serine hydrolases

100 µl of protein extract (in 67 mM Tris–HCl, pH 7.5 buffer including 10 mM DTT) from 24 h imbibed, non-aged, Arabidopsis seeds was first incubated with 50 µl streptavidin sepharose beads (GE Healthcare) for 1 h with constant mixing on a rotator at room temperature in order to remove the excess of biotinylated background proteins. After incubation, samples were centrifuged for 3 min at 1000×***g*** and the supernatant was collected in a clean tube, after which 1 volume of freshly prepared 10 M urea dissolved in extraction buffer was added. This mixture was then labelled with 2 µM ActivX™ Desthiobiotin-FP Serine Hydrolase Probe (ThermoFisher) for 2 h. An identical NPC was prepared following these instructions, but replacing the volume of probe with DMSO. After incubation, 50 µl of streptavidin sepharose beads was added and samples were incubated for 1 h with constant mixing on a rotator. After incubation, samples were centrifuged for 3 min at 1000×***g*** and the supernatant was collected in a clean tube. To test if the affinity enrichment worked, a part of the proteins were eluted by boiling in 2× SDS–PAGE loading buffer (5 min at 95°C). The rest of the beads were used for sample preparation for LC/MS/MS (on-bead digestion, OBD).

Sample preparation for LC/MS/MS

Affinity-enriched protein samples not eluted from the capture resin were on-bead digested (OBD). Briefly, streptavidin beads were washed twice with water to remove SDS. Then bound proteins were reduced with DTT (5 mM) in 50 mM ammonium bicarbonate (ABC) for 30 min at room temperature. Protein reduction was followed by alkylation with iodoacetamide (IAM, 10 mM also in 50 mM ABC, for 30 min at room temperature) and quenching of excess IAM with DTT (final concentration DTT 10 mM). Reduction and alkylation were followed by a sequential digestion of proteins with the first LysC for 3 h at 37°C followed by a 16 h digestion with trypsin at 37°C. The digestion was stopped by adding formic acid (FA) to a final concentration of 0.5%. The supernatant containing the digestion products was passed through home-made glass microfiber StageTip (GE Healthcare; pore size: 1.2 µm; thickness: 0.26 mm). The cleared lysates were then desalted on C18 StageTips.

Desalting and preparation for LC/MS/MS

Tryptic digests were desalted on home-made C18 StageTips as described [39]. Peptides were passed over a 2 disc StageTip. After elution from the StageTips, samples were dried using a vacuum concentrator (Eppendorf) and the peptides were taken up in 10 µl 0.1% FA solution.

LC/MS/MS

Experiments were performed on an Orbitrap Elite instrument (Thermo, [40]) that was coupled to an EASY-nLC 1000 liquid chromatography (LC) system (Thermo). The LC was operated in the one-column mode. The analytical column was a fused silica capillary (75 µm × 35 cm) with an integrated PicoFrit emitter (New Objective) packed in-house with Reprosil-Pur 120 C18-AQ 1.9 µm. The analytical column was encased by a column oven (Sonation) and attached to a nanospray flex ion source (Thermo). The column oven temperature was adjusted to 45°C during data acquisition. The LC was equipped with two mobile phases: solvent A (0.1% FA in water) and solvent B (0.1% FA in acetonitrile, ACN). All solvents were of UHPLC (ultra-high performance liquid chromatography) grade (Sigma). Peptides were directly loaded onto the analytical column with a maximum flow rate that would not exceed the set pressure limit of 980 bar (usually ∼0.5–0.8 µl/min). Peptides were subsequently separated on the analytical column by running a 140 min gradient of solvent A and solvent B (start with 7% B; gradient 7% to 35% B 120 min; gradient 35% to 100% B for 10 min and 100% B for 10 min at a flow rate of 300 nl/min.). The mass spectrometer was operated using Xcalibur software (version 2.2 SP1.48) and was set in the positive ion mode. Precursor ion scanning was performed in the Orbitrap analyser (FTMS; Fourier Transform Mass Spectrometry) in the scan range of *m/z* 300–1800 and at a resolution of 60 000 with the internal lock mass option turned on (lock mass was 445.120025 *m/z*, polysiloxane [41]). Product

ion spectra were recorded in a data-dependent fashion in the ion trap (ITMS; Ion Trap Mass Spectrometry) in a variable scan range and at a rapid scan rate. The ionisation potential (spray voltage) was set to 1.8 kV. Peptides were analysed using a repeating cycle consisting of a full precursor ion scan (3.0×10^6 ions or 50 ms) followed by 10 product ion scans (1.0×10^4 ions or 50 ms) where peptides are isolated based on their intensity in the full survey scan (threshold of 500 counts) for tandem mass spectrum (MS2) generation that permits peptide sequencing and identification. CID (collision-induced dissociation) collision energy was set to 35% for the generation of MS2 spectra. During MS2 data acquisition, dynamic ion exclusion was set to 120 s with a maximum list of excluded ions consisting of 500 members and a repeat count of one. Ion injection time prediction, preview mode for the FTMS, monoisotopic precursor selection and charge state screening were enabled. Only charge states higher than 1 were considered for fragmentation.

Peptide and protein identification using MaxQuant

Raw spectra were submitted to an Andromeda [42] search in MaxQuant (version 1.5.3.30) using the default settings [43]. Label-free quantification and match-between-runs were activated [44]. MS/MS spectra data were searched against the Uniprot *A. thaliana* reference database (UP000006548_3702.fasta, 33 439 entries, downloaded 2/9/2016). All searches included a contaminants database (as implemented in MaxQuant, 245 sequences). The database of contaminants contains known MS contaminants and was included to estimate the level of contamination. Enzyme specificity was set to 'Trypsin/P'. The instrument type in Andromeda searches was set to Orbitrap and the precursor mass tolerance was set to ±20 ppm (first search) and ± 4.5 ppm (main search). The MS/MS match tolerance was set to ± 0.5 Da. The peptide spectrum matches FDR and the protein FDR was set to 0.01 (based on target-decoy approach and decoy mode 'revert'). Minimum peptide length was 7 amino acids. Label-free protein quantification was switched on and unique and razor peptides were considered for quantification with a minimum ratio count of 2. Retention times were recalibrated based on the built-in nonlinear time-rescaling algorithm. MS/MS identifications were transferred between LC–MS/MS runs with the 'Match between runs' option in which the maximal match time window was set to 0.7 min and the alignment time window set to 20 min. The quantification is based on the 'value at maximum' of the extracted ion current. Modified peptides were allowed for quantification. The minimum score for modified peptides was 40. Further analysis and filtering of the results were done in Perseus (version 1.5.5.3, [45]).

Results

ABPP assays were conducted on artificially aged seeds from the Arabidopsis accessions Col-0 and L*er*. The ageing treatment was performed by incubating seeds for different periods at 37°C and 85% RH (see Materials and methods). After treatment, the seed viability level of these batches was determined in a germination assay. We observed that seed germination rapidly decreased with the ageing treatment, being reduced to only 10–20% after 8 days and rendering seeds were unable to germinate after 12 days (Figure 1). To study the behaviour of different proteases during the ageing process, we labelled protein extracts from these artificially aged seeds with four different probes targeting distinct classes of proteases: the proteasome, VPEs, papain-like cysteine proteases (PLCPs) and serine hydrolases (SH) (see Table 1). We did not observe changes in the labelling between non-aged and aged in protein extracts isolated from dry seeds (Supplementary Figure S1). Arabidopsis seeds are orthodox, meaning that their water content is dramatically reduced during the late phases of seed maturation [46]. In this dry state, the cytoplasm adopts a glassy conformation, which strongly decreases molecular mobility and could explain why we did not observe differences in the labelling pattern. Therefore, we conducted a second set of experiments using seeds imbibed for 24 h after the ageing treatment. In contrast with dry seed isolates, imbibed seeds showed differential labelling (Figures 2 and 5; Supplementary Figure S2). We observed that probe MV151 displayed a differential labelling in aged L*er* seeds. Conversely, this labelling pattern was not observed for Col-0 seeds, which is in agreement with their higher viability after artificial ageing (Supplementary Figure S2A). Specifically, labelling of the proteasome becomes more prominent in L*er* seeds during the ageing treatment (Supplementary Figure S2B), whereas PLCPs lose activity after accelerated ageing (Supplementary Figure S2C).

VPEs become active during seed ageing

VPEs [23,24] are cysteine proteases responsible for the processing and maturation of seed storage proteins [35,47]. Four VPE-encoding genes (α, β, γ and δ) have been described in Arabidopsis as being involved in plant immune responses [23,48], stress responses [49] and development [50,51].

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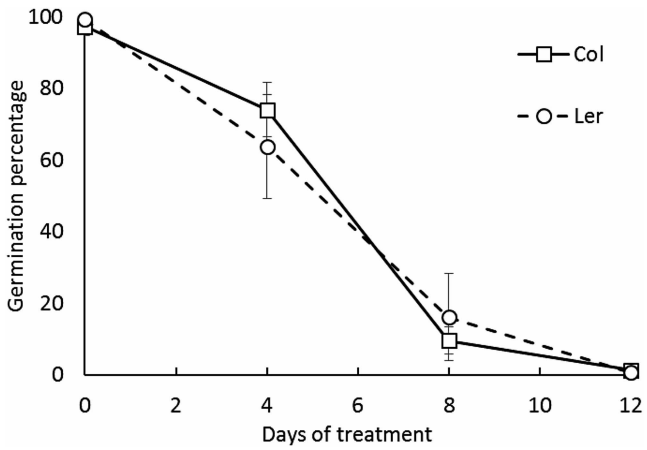

Figure 1. Germination proportion after different periods of accelerated seed ageing of seed lots from L*er* and Col-0 accessions.

Seed germination was evaluated after 0, 4, 8 and 12 days of accelerated ageing treatment (incubated at 37°C and 85% RH). Each data point represents the mean of at least three biological replicates. Error bars represent standard deviation.

To study the activity of VPEs in aged seeds of Arabidopsis, we labelled protein extracts from 24 h-imbibed seeds with JOPD1, in both Col-0 and L*er* accessions. Detection of fluorescence in these samples revealed several bands per sample. These bands showed an increase in intensity and the ageing treatment had been applied for long (Figure 2; Supplementary Figure S3). The observed bands of different sizes probably correspond to different maturation stages of the enzymes, as VPEs are first produced as zymogens which undergo an activation process through maturation. The intermediate form containing an N-terminal propeptide is already active and can be labelled as well as the mature form [23,52]. Previous studies have described βVPE and δVPE molecular sizes as two bands at 27 and 37 kDa and 38 kDa bands, respectively [51,53]. αVPE and γVPE were described as ranging from 40 to 46 kDa [23,24,54], which is comparable to the bands we observed at 38 kDa.

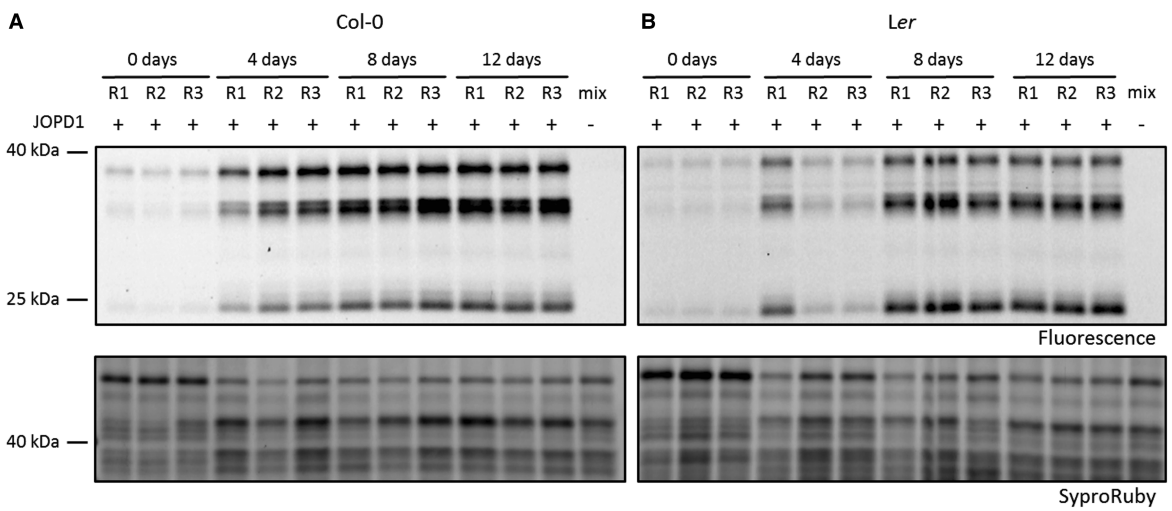

Figure 2. VPEs become active after artificial ageing.

Fluorescence detected in protein extracts from Arabidopsis Col-0 (**A**) and L*er* (**B**) accessions labelled with 1 μM probe JOPD1 (+) for 2 h at room temperature. For each time point of the treatment (0, 4, 8 and 12 days), three biological replicates (R1, R2 and R3) are shown (each protein isolate was extracted from 20 mg of seeds). The mix sample (mix) was prepared combining aliquots of all isolated proteomes and was used for the non-labelled sample (–). The bottom section in each panel corresponds to total protein loaded stained with SyproRuby. Values on the left side indicate molecular mass. Germination data corresponding to these seed samples is shown in Figure 1.

To determine which VPE activity is enhanced during the treatment, we evaluated seeds from different *vpe* mutant lines [35]. These mutants were first analysed for seed longevity phenotypes. Seeds from the *vpe* mutants and their wild-type Col-0 were grown and harvested at the same time. After storage to allow the release of seed dormancy, the seeds were artificially aged and their germination behaviour was scored. The mutant seeds did not show statistical differences from the wild type, although both β-*vpe* and the quadruple *vpe* mutant were slightly more sensitive to the ageing treatment (Figure 3A). Comparison of the wild-type JOPD1 labelling (Col-0) to that of the α, β and ɣ single *vpe* mutants confirmed that VPEs overlap and run at similar sizes in a gel (Figure 3B). Interestingly, the bottom-most band at 26 kDa seems to correspond to βVPE, as it is absent from all mutants lacking β*VPE*. Lane 6 displays the labelling of only ɣVPE, as this triple mutant lacks the other three VPEs. The combination of labelling in lanes 2, 5 and 6 seems to indicate that αVPE would correspond to the relatively thick uppermost band at 38 kDa. As expected, lane 7, corresponding to the quadruple *vpe* mutant, showed no labelling. Overall, we could not determine from these results whether a single VPE or several of them were responsible for the increased activity during the ageing treatment, especially considering both intermediate and mature forms are active and labelled, as previously shown [24]. However, the labelling observed in the different mutants suggests that more than one become active as a result of artificial ageing.

A previous publication described how γVPE becomes active after heat stress as part of the signalling pathway leading to programmed cell death (PCD) [55]. Specifically, this study described how heat treatments of 1 h at 40°C caused significant increases in γVPE activity. These conditions are similar to those we used for the accelerated ageing of seeds (several days at 37°C). To verify that the observed activation pattern reflects the effect of accelerated ageing but not a response to high temperature, the labelling pattern of naturally aged seeds was evaluated. Specifically, seeds naturally aged for 8 years from L*er* and *delay of germination 1* (*dog1-1*) mutant [56] were used to confirm our previous results. The *dog1-1* mutant loses seed viability faster than the wild type due to its reduced seed longevity [56,57]. A germination kinetics assay was conducted to determine the quality level of these seed batches (Figure 4A). L*er* seeds germinated up to 40%, whereas the longevity-deficient mutant *dog1-1* was unable to germinate. The comparison of labelled protein isolates from fresh, viable seeds and those from naturally aged seeds showed that the activation of VPEs observed after artificial ageing is also

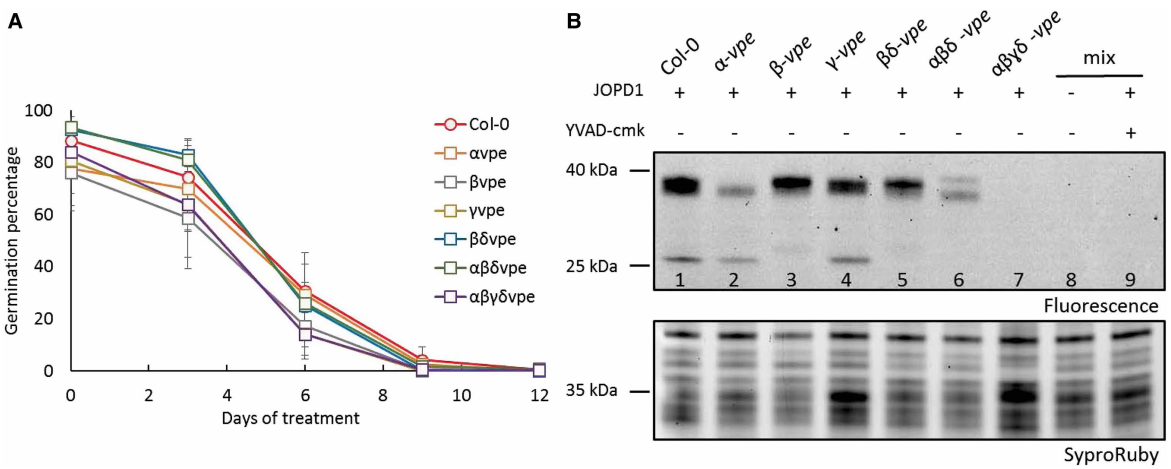

Figure 3. Seed longevity phenotype and labelling profile of Col-0, α, β, ɣ, βδ, αβδ and αβɣδ*vpe* mutants.

(**A**) The germination proportion of *vpe* mutants after different periods of artificial seed ageing (0, 4, 8 and 12 days). Each data point represents the mean of five biological replicates. Error bars represent the standard deviation. (**B**) The fluorescence detected in protein extracts from 8-day-aged seeds of Col-0 and the indicated *vpe* mutants labelled with 1 μM JOPD1 (top panel, +) and the total protein loaded stained with SyproRuby (bottom panel). Each protein isolate was extracted from 20 mg of seeds. The mix sample (mix) was prepared combining aliquots of all isolated proteomes and was used for the background labelling controls, the non-labelled sample (–) and the inhibitor control. The inhibitor used was YVAD-cmk at 50 μM (+). The bottom section in (**B**) corresponds to total protein loaded stained with SyproRuby. Values on the left-hand side of (**B**) indicate molecular mass.

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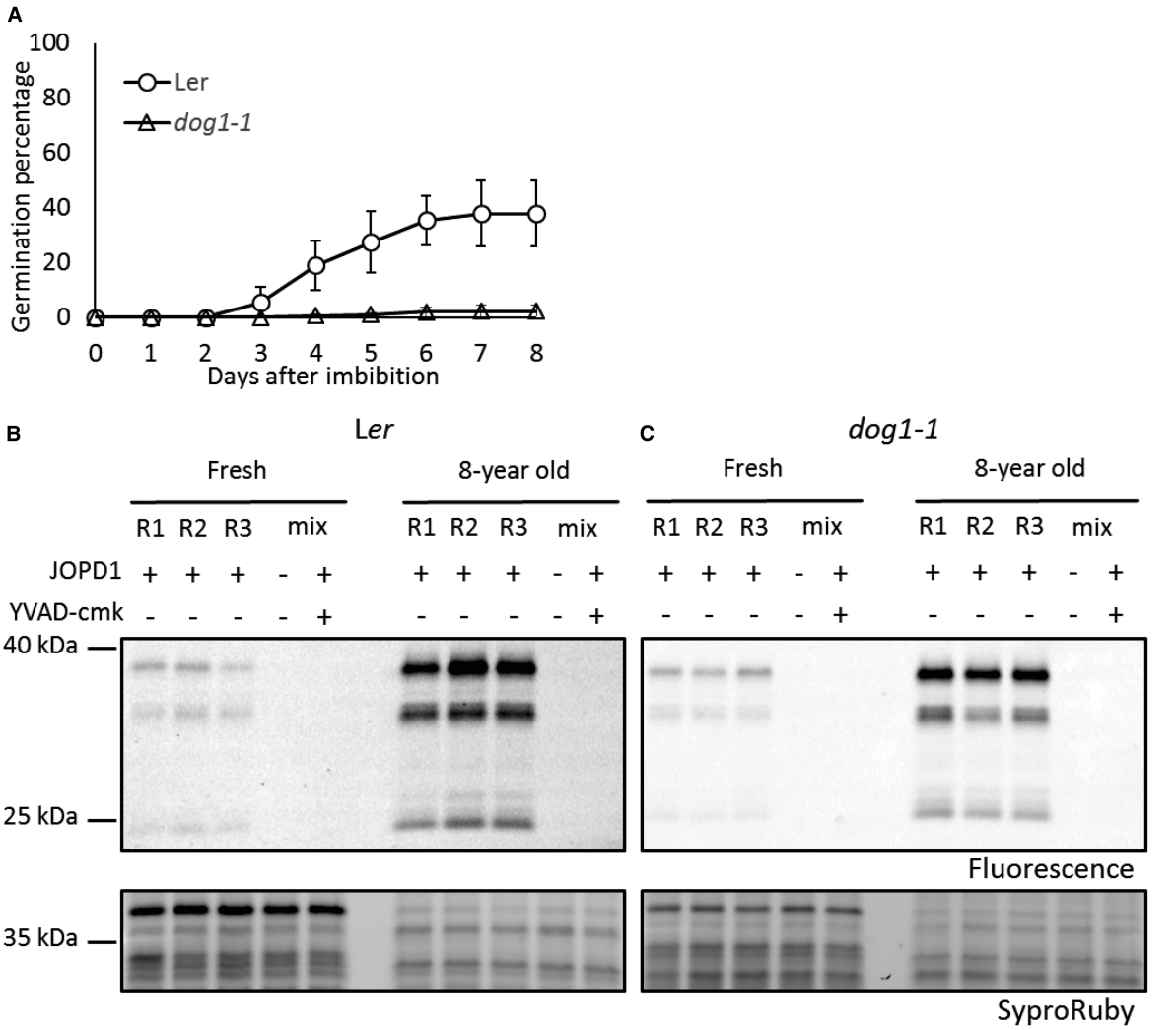

Figure 4. VPEs show increased activity in naturally aged seeds.

(**A**) The germination kinetics of 8-year-old naturally aged seed batches of L*er* and *dog1-1*. Each data point represents the mean of three biological replicates. Error bars correspond to standard deviation. (**B** and **C**) The fluorescence of JOPD1-labelled (1 μM) protein extracts (each protein isolate was extracted from 20 mg of seeds) from fresh and 8-year-old seeds of L*er* (**B**) and *dog1-1* (**C**). In each panel, three biological replicates (R1, R2 and R3) are shown from either fresh (left-hand side) or naturally aged (right-hand side) seeds. The mix sample (mix) was prepared combining aliquots of all isolated proteomes and was used for the background labelling controls, the non-labelled sample (NPC) and the inhibitor control. The inhibitor used was YVAD-cmk at 50 μM. The bottom section of (**B**) and (**C**) corresponds to the total protein loaded stained with SyproRuby. Values on the left side of (**B**) and (**C**) indicate molecular mass.

maintained in naturally aged samples (Figure 4B,C). In addition, gels of fresh and aged samples showed that the seed protein content also changes during the ageing process (Figure 4B,C, bottom panels) and that the *dog1-1* mutant displays an altered protein profile in comparison with L*er*, as observed in total protein staining. Taken together, our results confirm that VPEs increase their activity as a result of both natural and artificial ageing, making them good candidates as markers of seed quality.

Serine hydrolases become inactive as a result of ageing

Fluorophosphonate (FP)-based probes bind the active site of serine hydrolases, which are hydrolytic enzymes involved in a wide range of physiological processes, including development, defence and homeostasis [37]. Their contribution to such a variety of processes also relates with a broader distribution in different cell

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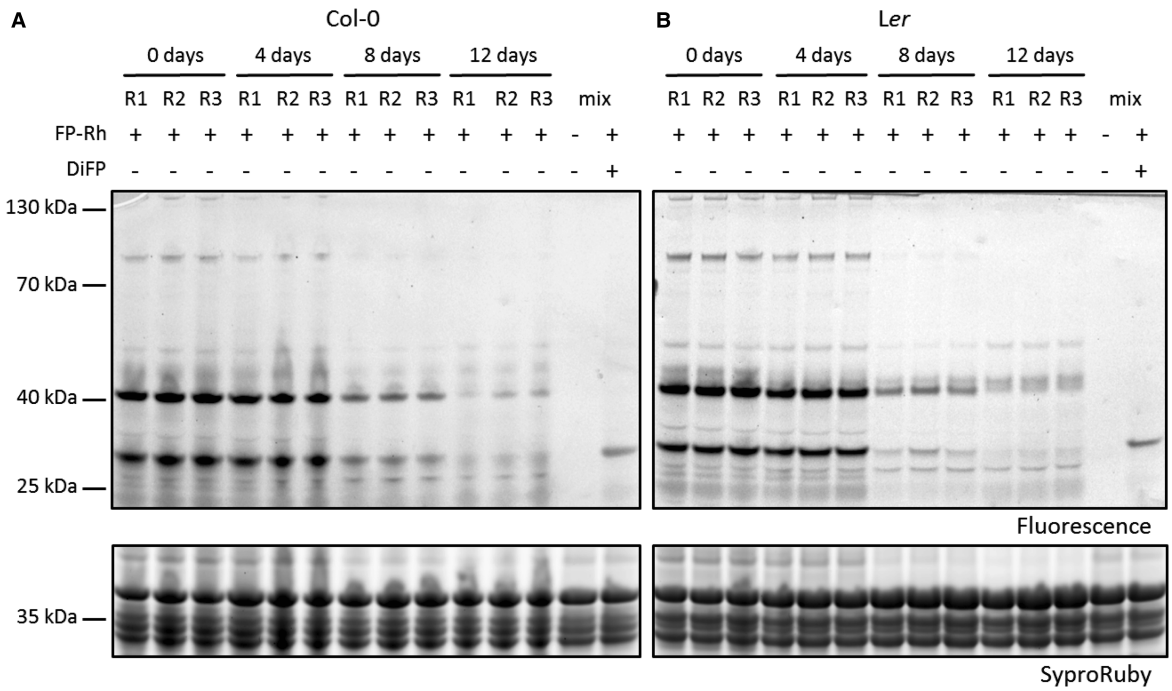

Figure 5. Serine hydrolases turn inactive during artificial ageing.

Detected fluorescence of protein extracts (each protein isolate was extracted from 20 mg of seeds) from Arabidopsis Col-0 (**A**) and L*er* (**B**) accessions labelled with 0.2 μM FP-Rh. For each time point, three biological replicates (R1, R2 and R3) are shown. The mix sample (mix) was prepared combining aliquots of all isolated proteomes and was used for the background labelling controls, the non-labelled sample (NPC) and the inhibitor control. The inhibitor used was DiFP at 100 μM. The bottom section in each panel corresponds to total protein loaded stained with SyproRuby. Values on the left-hand side indicate molecular mass. Germination data corresponding to these seed samples are shown in Figure 1.

compartments, including the cytoplasm and other organelles. This group of enzymes comprises more than 200 members in Arabidopsis and their activity is tightly regulated by several mechanisms, such as post-translational modifications and the presence of cofactors or inhibitors. Unlike the probes targeting VPEs, FP probes have a broader range of targets. Extracts from artificially aged seeds from Col-0 and L*er* imbibed for 24 h were labelled using FP-Rhodamine (FP-Rh, Table 1). In this case, multiple bands ranging from less than 20 kDa to sizes ∼130 kDa were observed in both accessions, including two stronger signals at ∼40 and 30 kDa (Figure 5). Interestingly, the intensity of two high-molecular mass bands at 100 and 135 kDa severely decreases after 8 days of treatment, similarly to the lower bands at ∼40 and 30 kDa that showed strong labelling in non-aged samples and fainter signals after 8 and 12 days (Figure 5; Supplementary Figure S4). The decreased labelling could be caused by the ageing treatment damaging these enzymes, thus rendering them inactive and unable to bind the probe. These results indicate that serine hydrolase activities gradually decline during prolonged artificial ageing treatment, in both Col-0 and L*er* accessions.

We next performed a pull-down assay to identify the FP-labelled proteins. Protein extracts of non-aged seeds were labelled with a biotin-tagged FP probe followed by streptavidin bead pull-down to purify labelled proteins, which were then submitted for MS analyses. Non-aged seeds were used to maximise the number of bands isolated, as they become fainter during ageing (Figure 5; Supplementary Figure S4). We used OBD to identify different proteins bound to the beads and determine their abundance in a quantitative approach. OBD analysis showed nineteen candidate hydrolases enriched in comparison with the NPC. Eighteen out of nineteen detected candidates are potential targets of the FP probe and correlate with hydrolase activity based on their Pfam domains (Figure 6). We observed some carboxylesterases and methylesterases also present in our NPC sample (Supplementary Dataset 1, putative candidates) indicating high abundance of those hydrolases in non-aged seeds. Besides, we found enrichment for one non-catalytic proteasome subunit (PBC2), although it is not a canonical target of the FP probe.

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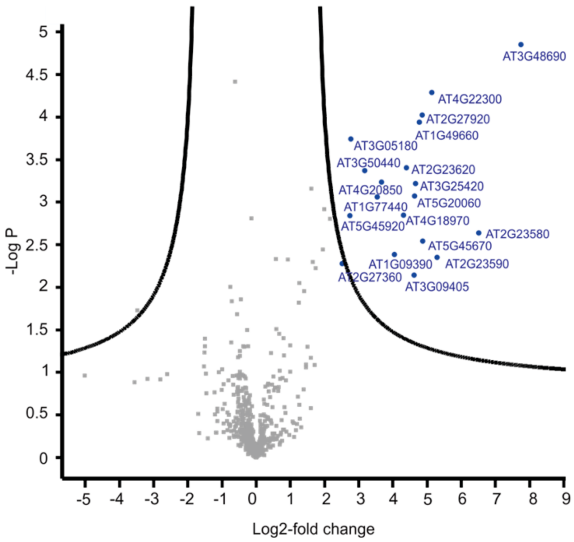

Locus	ID	Pfam
AT4G18970	GDSL esterase/lipase	Lipase GDSL
AT4G20850	Tripeptidyl-peptidase 2	Peptidase S8; TPPII
AT2G23590	Methylesterase 8	Abhydrolase 6
AT2G23580	Methylesterase 3	Abhydrolase 6
AT1G09390	DSL esterase/lipase	Lipase GDSL
AT1G77440	Proteasome subunit beta type-3-B	Proteasome
AT2G27920	Serine carboxypeptidase-like 51	Peptidase S10
AT5G45920	GDSL esterase/lipase	Lipase GDSL
AT4G22300	Carboxylesterase TIPSY-1	Abhydrolase 2
AT3G25420	Serine carboxypeptidase-like 20	Peptidase S10
AT5G20060	Phospholipase/carboxylesterase	Abhydrolase 2
AT2G23620	Methylesterase 1	Abhydrolase 6
AT3G50440	Methylesterase 10	Abhydrolase 6
AT5G45670	GDSL esterase/lipase	Lipase GDSL
AT1G49660	Putative carboxylesterase 5	Abhydrolase 3
AT3G05180	GDSL esterase/lipase	Lipase GDSL
AT3G48690	Putative carboxylesterase 12	Abhydrolase 3
AT3G09410	Pectin acetylesterase 5	PAE
AT2G27360	GDSL esterase/lipase	Lipase GDSL

Figure 6. Identification of FP-labelled candidates.

FP-labelled and non-labelled protein extracts from non-aged (0 days) seeds were compared using OBD to identify enrichment of serine hydrolases. Panel on the left shows a volcano plot highlighting significant differentially enriched proteins in the FP-labelled sample (blue, FDR = 0.05; SF = 1). Non-differentially enriched hits are shown in grey. Right panel shows the identity of putative proteins and their corresponding Pfam domain (https://pfam.xfam.org/).

Taken together, our results confirm ABPP as a valid approach to evaluate seed viability, which can aid towards determination of overall seed quality. Using this experimental approach, two different markers with opposite behaviour were identified. Specifically, the activity of VPEs was demonstrated to be induced by both artificial and natural seed ageing, whereas serine hydrolases exhibited the opposite trend, losing their activity during the treatment.

Discussion

In the present study, we have demonstrated the feasibility of activity-profiling of proteases as a tool to monitor levels of seed quality in seeds of *A. thaliana*. To this end, we used artificially aged seeds from the Arabidopsis accessions Col-0 and L*er* and looked for differential labelling in protein extracts from these seeds. Seed germination after different periods of artificial ageing was used as a proxy for seed viability, considering artificial ageing reduces seed vigour and overall seed quality [1,19].

ABPP probes are designed to covalently bind the active site of target enzymes in an activity-dependent manner, usually by substrate-mimicking or using tagged inhibitors [27]. This means that if, for any reason, the protein loses its conformation or the active site is blocked, labelling will not occur. In this work, we evaluated the activity-dynamics of several protease families during artificial seed ageing, as they are involved in a wide array of processes within the cell which can be affected by seed deterioration [32].

We evaluated four biochemical probes targeting different subsets of proteins and confirmed differential patterns of labelling in aged Arabidopsis seeds. The differential pattern in labelling intensities was only observed when seeds had been imbibed for 24 h prior to protein isolation and labelling, whereas labelled protein extracts from dry seeds showed no differences (Supplementary Figure S1). This indicates that imbibition and restoration of the metabolic activity of the cell are required to observe the changes in protein activity induced by artificial ageing. Probe MV151, which targets PLCPs and the 26S proteasome (Table 1) showed only a differential labelling pattern in aged L*er* seeds (Supplementary Figure S2). This differential labelling was not observed in Col-0, which is consistent with the germination data (Supplementary Figure S2A). In this experiment, the ageing treatment did not work properly in Col-0 seeds. The increase in proteasome activity during ageing fits with PCD events taking place at this stage (see below). Furthermore, the apparent loss of activity of PLCPs during the ageing treatment agrees with their roles in reserve mobilisation during seed germination [24]. However, further experiments will be required to confirm these results.

VPEs displayed a pattern of increased activity during the ageing treatment (Figure 2; Supplementary Figure S3). We showed that this increase in VPE activity also occurred in naturally aged seeds and, therefore, was not an artefact caused by artificial ageing (Figure 4). We tried to elucidate which VPE was responsible for the increased activity. However, we could not conclude which specific VPEs underlie the observed pattern from the labelling of artificially aged seeds of *vpe* mutants (Figure 3). Previous studies showed that VPEs have redundant functions in protein processing [33,35,53]. A compensation effect was also reported in PCD responses [48]. Mutants in δ-VPE, required for seed coat development, and even the quadruple *vpe* mutant were indistinguishable from the wild type [51]. It is therefore likely that redundancy also plays a role in the responses induced upon artificial ageing.

Interestingly, Asn-specific protein processing in developing Arabidopsis seeds occurs independently of VPE activity [35] and *vpe* quadruple mutants showed storage protein accumulation of alternatively processed polypeptides with altered solubility and protein assembly characteristics [33]. Although some studies have shown that VPEs are involved in protein processing upon seed germination [24,34,58] and heterologous overexpression of a *Vitis vinifera* VPE was shown to accelerate germination in Arabidopsis seeds [59], we did not see a significant effect on seed viability. Therefore, the lack of a seed longevity phenotype in the *vpe* mutants suggests that VPEs are not required in the acquisition of seed longevity.

The observed increase in VPE activity can arise from the production of hazardous compounds within the cells during seed ageing [60], which would act as a trigger to enhance VPE activity upon imbibition in order to process or isolate them into the vacuole [50,61]. Several studies have described that, apart from their role during seed maturation, VPEs play a role during PCD, both developmentally regulated [51] and as a result of pathogen attacks, including hypersensitive responses [61–63]. PCD processes can be elicited by vacuolar collapse [64] and VPE-silenced lines were shown to lack initiation of tonoplast disintegration and subsequent cell death [62]. The ageing process in seeds was also connected to PCD responses because DNA laddering, RNA degradation and membrane leakage were observed in aged seeds [60,65–68]. Given the role of VPEs as proteases, it is conceivable that they contribute to the turn-over of proteins damaged during the ageing process or that they become active as a part of PCD responses in response to ageing.

We observed that the activity of serine hydrolases is reduced during the ageing treatment in both Col-0 and L*er* accessions (Figure 5; Supplementary Figure S4). This is in agreement with previous reports that described the different damages incurred on the seed proteome during ageing [8]. As a result of the oxidative damage caused by seed ageing, protein denaturalisation and carbonylation occur. It is possible that the observed loss of activity is caused by these processes, although it remains to be confirmed experimentally.

Our FP pull-down assay of serine hydrolases identified eighteen different serine hydrolases in protein isolates from non-aged seeds compared with an identical non-labelled sample (Figure 6). Some of these targets have already been described as targets of FP probes and reported to be abundant proteins in plant tissues, for example CXE12 [37,69]. Interestingly, the size of CXE12 was shown to be close to 40 kDa, overlapping with one of the strongest signals we detected in non-aged seeds (Figure 5). Thus, we contemplate the possibility that this protein could be a potential serine hydrolase which has its activity reduced as a result of seed ageing. In addition, we identified several members of GDSL lipases, which have been reported to participate in lipid mobilisation during seed germination and early seedling development [70,71]. This enrichment of GDSL lipases is, therefore, in agreement with our protein extracts coming from 24-h-imbibed seeds. Evaluation of public transcriptomic data revealed that most of the identified hydrolases are expressed during either seed development or seed germination (Supplementary Figure S5A), which further supports their presence among our pull-down candidates. In most cases, it appears that these hydrolases are produced at earlier stages of seed development and stored in the dry seed, only to regain activity during seed germination, as for example At3g25420 (SCPL20) or At4g18970. Conversely, some of the identified candidates are highly expressed after 24 h of seed imbibition, which might explain their abundance at this stage and why they appeared enriched in the non-labelled fraction of our pull-down (Supplementary Figure S5B, NPC), for example At5g45920. Nevertheless, the relationship between transcript abundance and protein accumulation and activity is not direct, indicating many regulatory mechanisms are taking place, such as post-translational modifications. These observations support the use of novel tools, such as ABPP, to monitor changes in the active state of enzymes. Further work should address the biological meaning of these relationships and the putative role of the identified hydrolases in seed viability and overall quality.

Classically, the approaches used to evaluate seed storability and viability have relied on the use of germination tests combined with accelerated ageing treatments [19] or the use of a tetrazolium test, which stains only

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living tissues and allows quantification of living seeds [72]. During the years, several methods have been proposed to investigate seed quality. In *Brassica*, the fluorescence of sinapine was used to distinguish dead seeds from viable ones [73]. Likewise, the abundance of HSP17.6I was positively correlated with seed performance in this species [74] and changes in enzyme activities in four *Brassica* species were proposed as indicators of seed deterioration [75]. Similarly, several studies have proposed addressed the use of novel seed quality markers, such as the production of methanol, ethanol or galactinol in stored seeds [11,12,76–78]. However, the development of novel, equally reliable methods is always desirable. This is particularly relevant when recent reports question the suitability of classical ageing methods as predictive tools of seed longevity [79].

In this study, we report the use of ABPP as a tool to evaluate seed viability. We have identified different hydrolase activities which exhibit opposite trends during seed ageing. Besides, we have identified eighteen potential serine hydrolase candidates which might lose their activity as a result of seed ageing. We propose that the combined analysis by ABPP of protease activities has the potential to be developed into an easy-to-use, high-resolution marker for seed quality.

Abbreviations

ABC, ammonium bicarbonate; ABPP, activity-based protein profiling; ACN, acetonitrile; CID, collision-induced dissociation; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FA, formic acid; FDR, false discovery rate; FP, fluorophosphonate; FP-Rh, FP-rhodamine; FTMS, Fourier transform mass spectrometry; IAM, iodoacetamide; ITMS, ion trap mass spectrometry; LC, liquid chromatography; MS, mass spectrometry; NPC, no-probe control; OBD, on-bead digestion; PCD, programmed cell death; PLCPs, Papain-like cysteine proteases; RH, relative humidity; ROS, reactive oxygen species; SH, serine hydrolases; UHPLC, ultra-high performance liquid chromatography; VPEs, vacuolar processing enzymes.

Author Contribution

N.V.T. wrote the manuscript with input from all authors. N.V.T., R.A.L.H., W.J.J.S. and J.C.M.V. designed the experiments. N.V.T. and J.M.V. performed the experiments. F.K. and M.K. performed the mass spectrometry experiments.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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