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Research article

Subtilase activity and gene expression during germination and seedling growth in barley

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ABSTRACT

Proteases play a main role in the mobilization of storage proteins during seed germination. Until today, there is little information about the involvement of serine proteases, particularly subtilases, in the germination of barley grains. The aims of the present work were to study the contribution of serine proteases to the total proteolytic activity induced during germination of barley grains and evaluate the specific involvement of subtilases in this process. Proteolytic activity assayed against azocasein in the presence of specific inhibitors, showed that serine proteases contributed between 10 and 20% of total activity along germination. Subtilase activity increased from day 1 after imbibition with a peak between days 4–5. Moreover, *in vivo* determination of subtilase activity in germinating grains revealed increasing activity along germination mainly localized in the seed endosperm and developing rootlets. Finally, the expression of 19 barley genes encoding subtilases was measured by real time PCR during germination. Three of the analyzed genes increased their expression along germination, five showed a transient induction, one was down-regulated, nine remained unchanged and one was not expressed. The present work demonstrates the involvement of subtilases in germination of barley grains and describes the positive association of eight subtilase genes to this process.

1. Introduction

In a strict sense, germination starts with seed imbibition and ends when radicle emerges through the seed coat. However, many authors have employed the term germination in a broader sense including first period of seedling growth as part of the germination phase. In agreement with the vast literature, in the present work we have preferred this latter definition.

Embryo development is initially supported by nutrients accumulated in seeds, being nitrogen provided mostly by degradation of storage proteins. Major storage proteins in barley grains are prolamins named hordeins and are located in the cells of the endosperm forming a dense matrix that surrounds the starch granules. During the germination of barley grains, hordeins are hydrolyzed by proteolytic enzymes into simple molecules such as amino acids and peptides, essentials for germination and seedling development.

In barley, germination associated proteolysis is also relevant to the industrial processing of grains. Both, insufficient and excessive hydrolysis of storage proteins can affect malt and beer quality (Osman et al., 2002).

To date, the spectrum of proteolytic enzymes involved in storage protein degradation during germination remains unresolved, although participation of proteases from all the different classes have been proven or suggested (Jones and Budde, 2005). Zhang and Jones (1995) reported the presence of more than 40 different protease actives in germinating barley grains identified by two-dimensional gel electrophoresis. According to the authors, four of the observed activities corresponded to aspartic proteases, three to metalloproteases, eight to serine proteases and 27 to cysteine proteases.

Cysteine proteases are not only the most abundant but also the most studied proteases in germinating barley grains. Two cysteine proteases, EP-A and EP-B, have been demonstrated to hydrolyze B- and D-hordein

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DAI, days after imbibition; DMSO, dimethyl sulfoxide; DTT, 1,4-dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; EU, enzymatic unit; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; FW, fresh weight; LSD, Fisher's Least Significant Difference; PMSF, phenylmethylsulfonyl fluoride; pNA, p-nitroaniline; Suc-AAPF-pNA, Ala-Ala-Pro-Phe-p-nitroanilide; TCA, trichloroacetic acid; TPCK, N-*p*-tosyl-L-phenylalanine chloromethyl ketone; TPPE, thionin proprotein-processing enzyme

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into small peptides (Koehler and Ho, 1988, 1990) and are indicated as the main responsible for storage protein degradation during grain germination. In addition, a role of cysteine proteases in the release of bound *beta*-amylase during barley germination (Sopanen and Laurière, 1989) and in *beta*-amylase activation through partial proteolysis of a pre-protein with low activity (Guerin et al., 1992) has been demonstrated. Other authors have shown the correlation between a cysteine protease gene expression and malting quality traits except for *beta*-amylase activity (Potokina et al., 2004).

While it has been suggested that serine proteases play no significant role in storage protein solubilisation, at least during mashing (Jones and Budde, 2005), different authors have demonstrated consistently increased gene expression of several serine proteases during germination (Sreenivasulu et al., 2008; Lapitan et al., 2009; An and Lin, 2011), and in some cases correlation with malting quality traits has been found (Potokina et al., 2004, 2006). In addition, protein spots exhibiting serine protease activity have been identified in 4-day germinated barley seeds (Zhang and Jones, 1995). Dal Degan et al. (1994), reported protein and gene expression of different carboxypeptidases of the serine type in the embryo and aleurone of germinating barley grains, and some of them were shown to be secreted into the endosperm. Although *in vivo* substrates for barley grain serine proteases are still unknown, degradation of *beta*-amylase by several enzymes present in green malt extract and belonging to the serine-class has been shown *in vitro* (Schmitt and Marinac, 2008).

Among serine proteases, those belonging to family S8 (Rawlings et al., 2018), also known as subtilisin-like endopeptidase or subtilase family, have been specifically associated to the germination process, both at protein and gene expression level. So far, two distinct subtilases, named hordolisin (Terp et al., 2000) and SEP-1 (Fontanini and Jones, 2002) have been isolated from green malt. Both subtilases show increasing activity along germination but since they were not able to hydrolyzed hordein preparations *in vitro* and their presence in the starchy endosperm has not been detected (Terp et al., 2000; Fontanini and Jones, 2002), a role in controlling some other aspects of grain germination, different from reserves mobilization, can be expected for these enzymes. However, gene expression of at least two subtilase genes (Contig 13847_s_at and Contig 20186_at) has been shown to increase during germination (Schmitt et al., 2013), and one of them (Contig 13847_s_at) was reported to correlate positively with malt extract and negatively with malt beta-glucan content in more recently developed barley varieties compared to older ones (Muñoz-Amatriaín et al., 2010). Therefore, an indirect effect on storage protein mobilization cannot be discarded.

Considering that the role of serine proteases, and in particular that of subtilases, in the germination process of barley grains remains unresolved, the aim of this work was to study the contribution of serine proteases to the total proteolytic activity induced during germination of barley grains and, more specifically, to evaluate the involvement of subtilases in this process. In order to identify candidate subtilases relevant to the germination process, the expression of several barley subtilase genes was examined in germinating grains.

2. Materials and methods

2.1. Plant material

Barley seeds (*Hordeum vulgare* cv. Scarlett) were surface-sterilized with 0.3% (w/v) NaOCl solution for 20 min and washed several times with sterile distilled H_2O. Grains were then steeped in distilled water at 19 °C for 36 h with two 1 h-air rests. After steeping, grains were allowed to germinate on filter paper soaked with distilled H_2O in the dark at 22 °C and samples were collected every 24 h during 5 d. Germinating grains were dissected from non-adherent tissues (rootlets and coleoptiles), frozen immediately in liquid nitrogen and stored at −80 °C. For evaluation of germination rate and seedling development, the percentage of germinated grains and the length of coleoptile and rootlets were recorded daily until 5 days after imbibition (DAI).

2.2. Extract preparation

Grain samples were ground with a manual grinder and liquid nitrogen until obtaining a fine powder. Soluble fraction was extracted [3.3 ml buffer g^{-1} fresh weight (FW)] with 50 m*M* sodium acetate buffer (pH 5.0), containing 1 mM DTT (1,4-dithiothreitol). Homogenate was centrifuged at 15000 × g for 30 min at 4 °C. Supernatant was used for subsequent determinations.

2.3. Protein and free amino acids determination

Soluble proteins in the supernatant were determined according to Bradford (1976) using bovine serum albumin (BSA) as standard. Free amino acids in the supernatant were measured with ninhydrin reagent according to Yemm and Cocking (1955) after precipitation of proteins with 5% (w/v) TCA (trichloroacetic acid). Standard curve for amino acid determination was constructed using glycine as standard.

2.4. Proteolytic activity

Total proteolytic activity was evaluated using azocasein (Sigma-Aldrich, USA) as substrate. An aliquot of 100 µl of supernatant was mixed with 50 mM sodium acetate buffer (pH 5.0), 60 µl of azocasein (25 mg ml^{-1} in $NaCO_3H$ 1%) in a final volume of 300 µl. A reaction blank without supernatant was included in the assay together with one control without substrate for each supernatant sample. The reaction mixture was incubated at 37 °C for 2 h and the reaction was stopped with 1.2 ml of 5% (w/v) TCA. Precipitation of denatured proteins was achieved by incubating the samples on ice for 15 min. After centrifugation at 15000 xg for 15 min, supernatant was recovered and the absorbance was measured at 340 nm. One enzymatic unit (EU) was defined as the amount of enzyme causing a 0.01 increase in A_{340} over the 0 time values obtained by adding the TCA solution immediately after the substrate.

In order to characterize the spectrum of proteases involved in the germination of barley grains the following inhibitors were used: 10 µM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µM chymostatin, 100 µM N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 5 mM 1,10-phenanthroline, 5 mM (ethylenedinitrilo)tetraacetic acid (EDTA), and 1 µM pepstatin A. Each protease inhibitor was added to the reaction mix together with the supernatant and pre-incubated at 37 °C for 20 min before the addition of the substrate. After that, reaction proceeded as described above. Hydrolysis of azocasein was expressed as the percentage of residual activity, considering as 100% the values measured in the absence of inhibitors but including in the reaction an equivalent volume of the corresponding inhibitor solvent: DMSO (dimethyl sulfoxide) for chymostatin, ethanol for PMSF and TPCK, and methanol for pepstatin A and 1,10-phenanthroline.

The method was modified when testing the effect of 1,10-phenanthroline due to its high absorbance at 340 nm. After protein precipitation with TCA and centrifugation, supernatant was transferred to a clean microtube and the pH was changed by the addition of sodium hydroxide (NaOH) to final concentration of 0.5 N. Samples with and without (control of 100% activity) 1,10-phenanthroline were measured spectrophotometrically at 440 nm.

Subtilase activity was assayed using the synthetic colorimetric substrate succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA, Sigma-Aldrich, USA). Reaction mixture consisted of 50 mM sodium phosphate buffer (pH 8.0), 5 mM Suc-AAPF-pNA dissolved in DMSO and an aliquot of the supernatant equivalent to 4.5 mg of FW. Samples were incubated at 37 °C for 1 h. The reaction was stopped by adding 2 M acetic acid. Free pNA (p-nitroaniline) was quantified at 405 nm with a

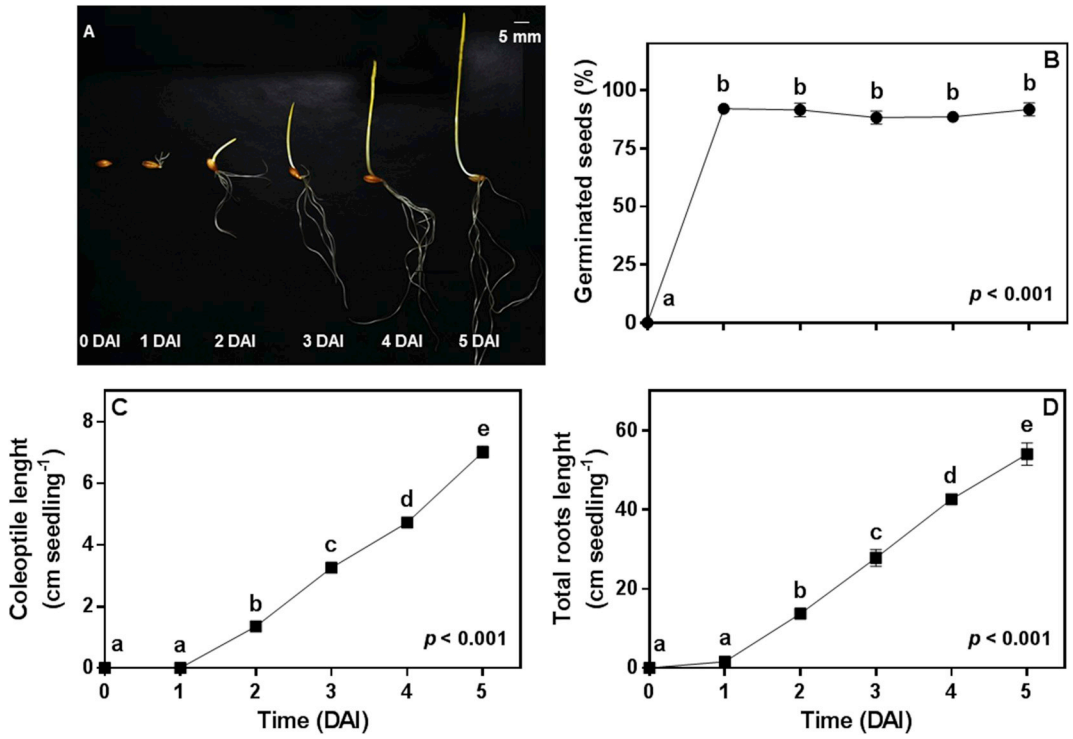

Fig. 1. Germination of barley grains (A), percentage of germinated seeds (B), and development of seedling coleoptile (C) and rootlets (D) from 0 to 5 days after imbibition (DAI). Data represent mean ± SE (n = 4). Different letters show significant differences with $p < 0.001$.

Multiskan® EX microplate reader (Thermo Fisher Scientific, USA). pNA molar extinction coefficient used for calculations was 9500 l mol^{-1} cm^{-1} at 405 nm. Effect of PMSF, chymostatin and E-64 on subtilase activity was assayed at the same concentrations mentioned above. The reaction mix containing the supernatant sample together with each one of the inhibitors was pre-incubated for 10 min prior to substrate addition.

2.5. Effect of PMSF on germination and seedling growth

Barley grains surface-sterilized as described above were placed in Petri dishes on top of two filter paper disks soaked with 4 ml of sterile distilled water (control) or a solution containing the following concentrations of PMSF: 100 µM, 200 µM, 500 µM or 1 mM. Petri dishes were incubated in germination chamber at 20 °C. Percentage of germinated grains was accounted per day and coleoptile and rootlets length was measured after 5 days.

2.6. Subtilase activity in vivo

Germination assay was performed as mentioned and germinated grains were collected at different times from 0 to 5 DAI. Grains were longitudinally sectioned and placed in a Petri dish on top of two layers of filter paper moistened with sterile distilled water. Ten microliters of 10 µM Suc-AAPF-pNA were applied to each half grain and plates were incubated at 30 °C in the dark for 30 min. After incubation, diazotization of free pNA was performed as previously described (Ohlsson et al., 1986). Briefly, the following solutions were added to the half seeds with an incubation period of 5 min between one solution and the next one: 0.5% sodium nitrite in 1 M HCl, 0.5% ammonium sulphamate in 1 M HCl and 0.05% *N*-(1-Naphthyl)ethylenediamine in 47.5% ethanol. Diazotization of free pNA rendered an intense pink dye indicative of efficient substrate hydrolysis. Controls without substrate but with an equal volume of substrate solvent (DMSO) were performed for each sampling time. Twenty germinated grains were tested for each sampling time. Seeds were photographed with a digital camera attached to a magnifying glass.

2.7. Total RNA extraction, cDNA synthesis and real time PCR

Total RNA was extracted from 100 mg FW of ground germinated grains (0–5 DAI) using TRIzol™ Reagent (Thermo Fisher Scientific, USA) and treated with RQ1 RNase-free DNase (Promega Corporation, USA). RNA integrity was examined by electrophoresis in agarose gels stained with SYBR™ Safe (Thermo Fisher Scientific, USA) and its concentration and purity was determined with NanoDrop™ (Thermo Fisher Scientific, USA). cDNA was synthesized from 1 µg of RNA using M-MLV™ Reverse Transcriptase (Thermo Fisher Scientific, USA) and Oligo (dt) according to manufacturer's instructions. cDNA samples were used as templates to analyze the expression of 19 subtilase genes by real time PCR. Actin and elongation factor-1-alpha were analyzed as reference genes. Primer sequences and gene information are provided as supplemental data (Supplemental Table S1). Amplification reaction was performed using the Fast Start Universal SYBR Green Master (ROX) from Roche (Roche, Argentina) and the Stratagene Mx3000P QPCR thermocycler (Agilent Technologies, USA). All primers were designed and tested to have similar Tm values allowing suitable amplification of all genes at the same temperature. The following amplification programme was used: 1 cycle of 10 min at 95 °C, 50 cycles of 95 °C for 15 s, 60 °C for 30 s, 60 °C for 1 min and a final cycle of 1 min at 95 °C, 30 s at 55 °C and 30 s at 95 °C. Comparative Ct (threshold cycles) method (ΔΔCt) was applied for relative quantification of gene expression using the software provided with the thermocycler.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed followed by Fisher's Least Significant Difference (LSD) test for post-hoc comparisons of means. *P*-values were considered significant when lower than 0.05. Four independent replicates were used for all the biochemical analysis and three for the molecular analysis. A Pearson correlation was carried out to analyze possible correlations between the measured parameters.

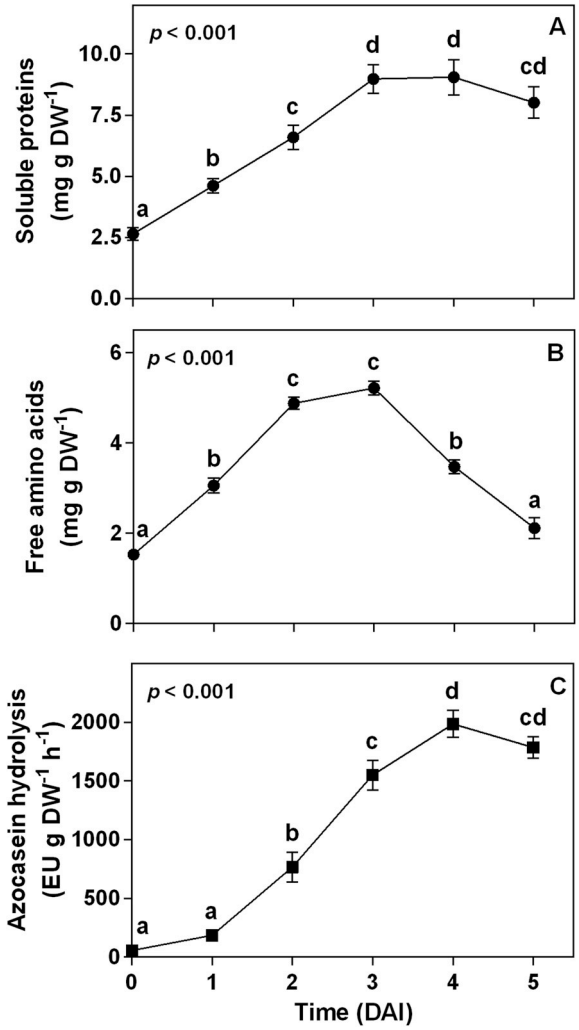

Fig. 2. Changes in soluble protein (A), free amino acids (B) and total protease activity normalized by seed dry weight (C) along germination of barley grains from 0 to 5 DAI. Data represent mean ± SE (n = 4). Different letters show significant differences with $p < 0.001$.

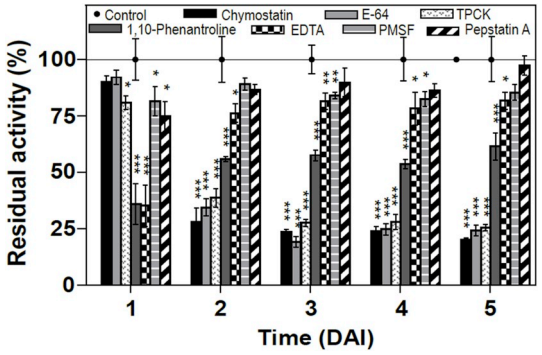

Fig. 3. Contribution of the different catalytic groups to the total proteolytic activity along germination. Activity was measured with azocasein as substrate and data is shown as residual activity respect to the control (no inhibitor) at each sampling time. Data represent mean ± SE (n = 4). Significant differences are indicated with one asterisk for $p < 0.05$; two asterisks for $p < 0.01$ and three asterisks for $p < 0.001$.

3. Results

3.1. Protein degradation during germination and seedling growth

The morphology of germinating barley grains at each sampling point is shown in Fig. 1A. Seeds showed high germination efficiency, reaching a maximum of 93% germinated grains during the first 24 h after imbibition (Fig. 1B). Once germination process was initiated, both coleoptile and the root system developed continuously along time (Fig. 1C and D).

Biochemical determinations were performed in soluble extracts prepared from whole grains after removal of coleoptile and emerging rootlets. In the assayed conditions, soluble protein concentration increased until day 3 of germination reaching a peak of 3.4 times the initial amount and then remained almost invariable until day 5 (Fig. 2A). In the same way, the free amino acid concentration increased until day 2 reaching a peak of 3.2 times the initial concentration, and then it decreased until day 5 (Fig. 2B). Total endoprotease activity measured as azocasein hydrolysis showed a sharp increase between days 1–4, remaining at the maximum level until the end of the assay (Fig. 2C). Highest azocaseinolytic activity represented values of about 35-fold the initial activity (day 0) when normalized by dry weight biomass (Fig. 2C).

The contribution of the different catalytic groups to the total proteolytic activity during germination was determined by the use of several protease inhibitors. Results are shown as residual activity with respect to a control without inhibitors for each sampling time (Fig. 3). Total protease activity was very low at day 0 and no significant inhibition was detected by any of the inhibitors assayed (not shown). Cysteine proteases, specifically inhibited by E-64, accounted for the highest fraction of azocasein hydrolysis during germination, determined as 65% of total activity at 2 DAI and between 80 and 75% the next sampling dates. The contribution of serine proteases represented between 20 and 10% of the total activity along the germination process as evidenced by PMSF. The use of TPCK or chymostatin revealed inhibition patterns very similar to E-64, being strongly significant since day 2. Contribution of metalloproteases, evidenced by both EDTA and 1,10-phenantroline, was highest at 1 DAI accounting for 65% of total activity. Then, it declined as germination progressed but remained significant until the end of the assay, varying between 20 and 40% of total azocaseinolytic activity. As for aspartic proteases, sensitive to pepstatin A, its activity was only significant at day 1 with a contribution of about the 25%, making it the group with the minor contribution to proteolytic activity during the germination phase (Fig. 3).

3.2. Barley grains germination is inhibited in the presence of PMSF

In order to deep into the involvement of serine proteases, an *in vivo* experiment was designed to evaluate the possible inhibition of barley germination by applying PMSF directly to the grains (Fig. 4). Germination rate was recorded every 24 h and the results showed that germination was affected by PMSF at concentrations of 200 µM or higher (Fig. 4A and Supplemental Figure S1). Germination was both delayed and reduced to 80% respect to the control when applying 200 µM PMSF and it was completely inhibited at 500 µM. Both, radicular and coleoptile development measured at day 5 of treatment were significantly reduced as compared to the control (Fig. 4B and C). The lowest PMSF concentration tested (100 µM) had no effect on germination rate and seedling growth. The same assay was performed using chymostatin as a cysteine and chymotrypsin-like proteases inhibitor without obtaining differences with respect to the control (data not shown).

3.3. Subtilase activity increases during the germination process

Activity of subtilases in germinating grains was analyzed with the synthetic peptide Suc-AAPF-pNA (Fig. 5). Subtilase activity increased throughout the assay until day 4 and remained high at day 5 reaching a peak of 12-fold the initial activity demonstrating that subtilases are active along germination in barley grains. Controls in the presence of E-

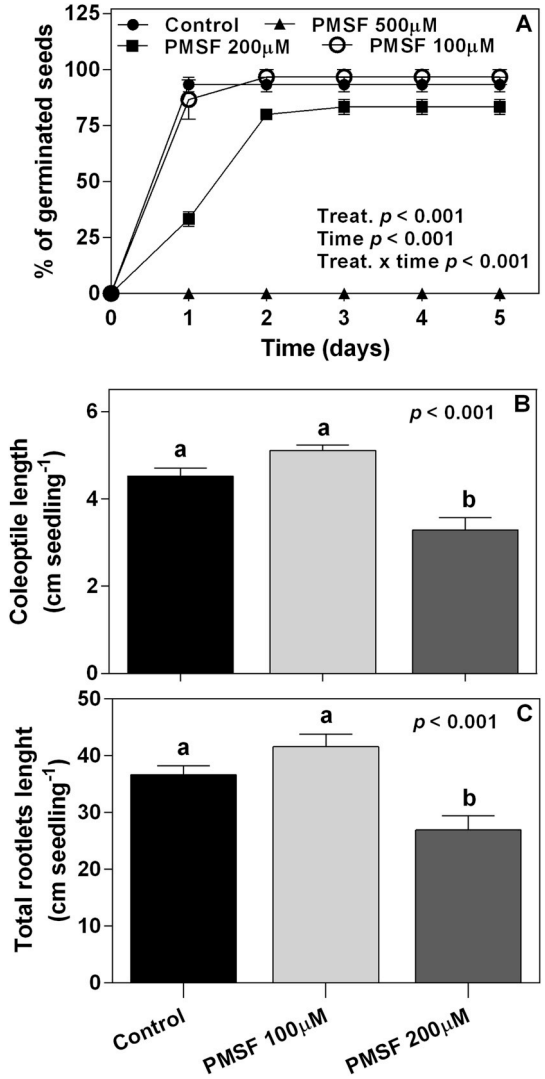

Fig. 4. Effect of PMSF on the germination of barley grains. (A) Percentage of germinated barley seeds in the presence of water (control) or different concentrations of PMSF from 100 to 500 μM along time. (B) Coleoptile and (C) total roots length of barley seedlings determined at day 5. Data represent mean ± SE (n = 3). Different letters show significant differences with $p < 0.001$.

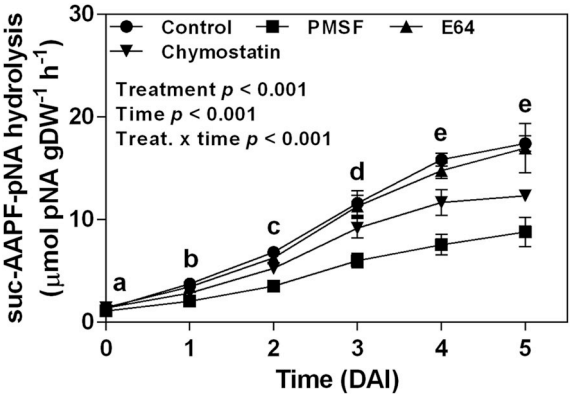

Fig. 5. Subtilase activity in soluble extracts of germinating barley grains from 0 to 5 DAI. Suc-AAPF-pNA hydrolysis measured in the absence of inhibitors (control) or in the presence of PMSF, E-64 or chymostatin. Data represent mean ± SE (n = 3). Different letters show significant differences with $p < 0.001$.

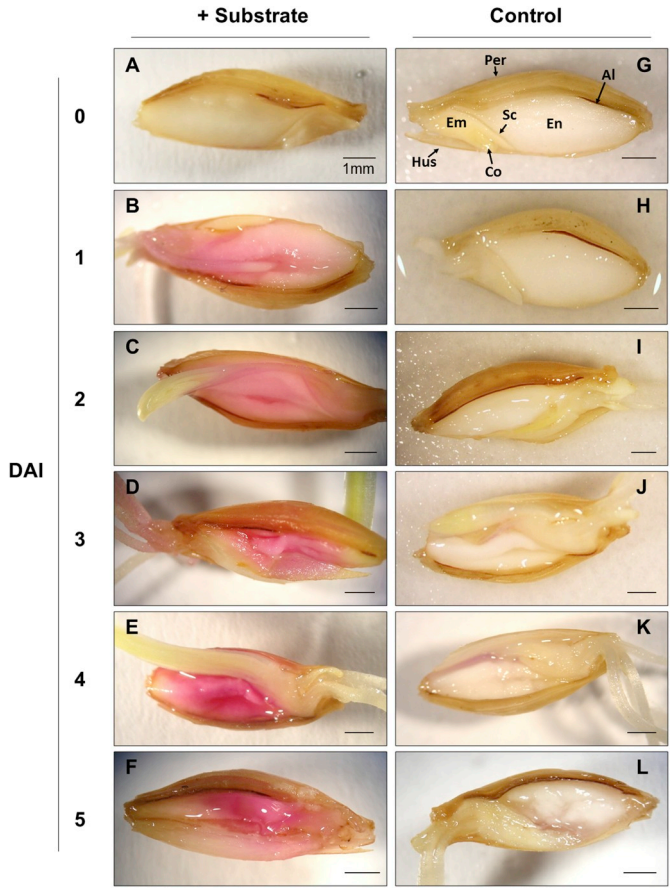

Fig. 6. *In vivo* determination of subtilase activity in germinating barley grains. Suc-AAPF-pNA was added to longitudinally sectioned barley grains. After incubation liberated pNA was revealed by diazotization as a bright pink dye (A to F). Controls without substrate but in the presence of DMSO (substrate solvent) are shown (G to L). En, endosperm; Al, aleurone layer; Sc, scutellum; Em, embryo; Co, coleoptile; Per, pericarp; Hus, husk (lemma and palea).

64, chymostatin and PMSF were conducted. Results showed no significant differences when applying E-64 with regard to the control and a strong inhibition by PMSF, demonstrating that the enzyme(s) responsible for substrate degradation are mainly serine proteases. The presence of chymostatin also lowered Suc-AAPF-pNA hydrolysis compared to the control but was not as efficient as PMSF (Fig. 5).

By using a protocol recently developed to visualize subtilases activity *in vivo* (Galotta and Roberts, 2019), we were able to confirm the occurrence of subtilase activity in germinating barley grains (Fig. 6). The method is based on the diazotization of p-nitroaniline (pNA) liberated from the synthetic substrate Suc-AAPF-pNA by the action of subtilases. Briefly, substrate is applied to germinating grains longitudinally sectioned which are then incubated to allow hydrolysis by subtilases. Free pNA is visualized as a bright pink coloration after diazotization. No substrate degradation was revealed at 0 DAI indicating null o very low subtilase activity (Fig. 6A). As germination progressed, both the embryo and endosperm acquired a pink colouration showing increasing intensity with time (Fig. 6B–F). In addition, high subtilase activity was detected in developing rootlets with this method (Supplemental Figure S2).

3.4. Induction of subtilase gene expression in germinating barley grains

In order to identify individual subtilases induced during germination of barley grains, expression of 19 subtilase genes was analyzed by real time PCR in samples collected every 24 h from 0 to 5 DAI (Fig. 7). Identification of 9 of the 19 genes analyzed was reported before

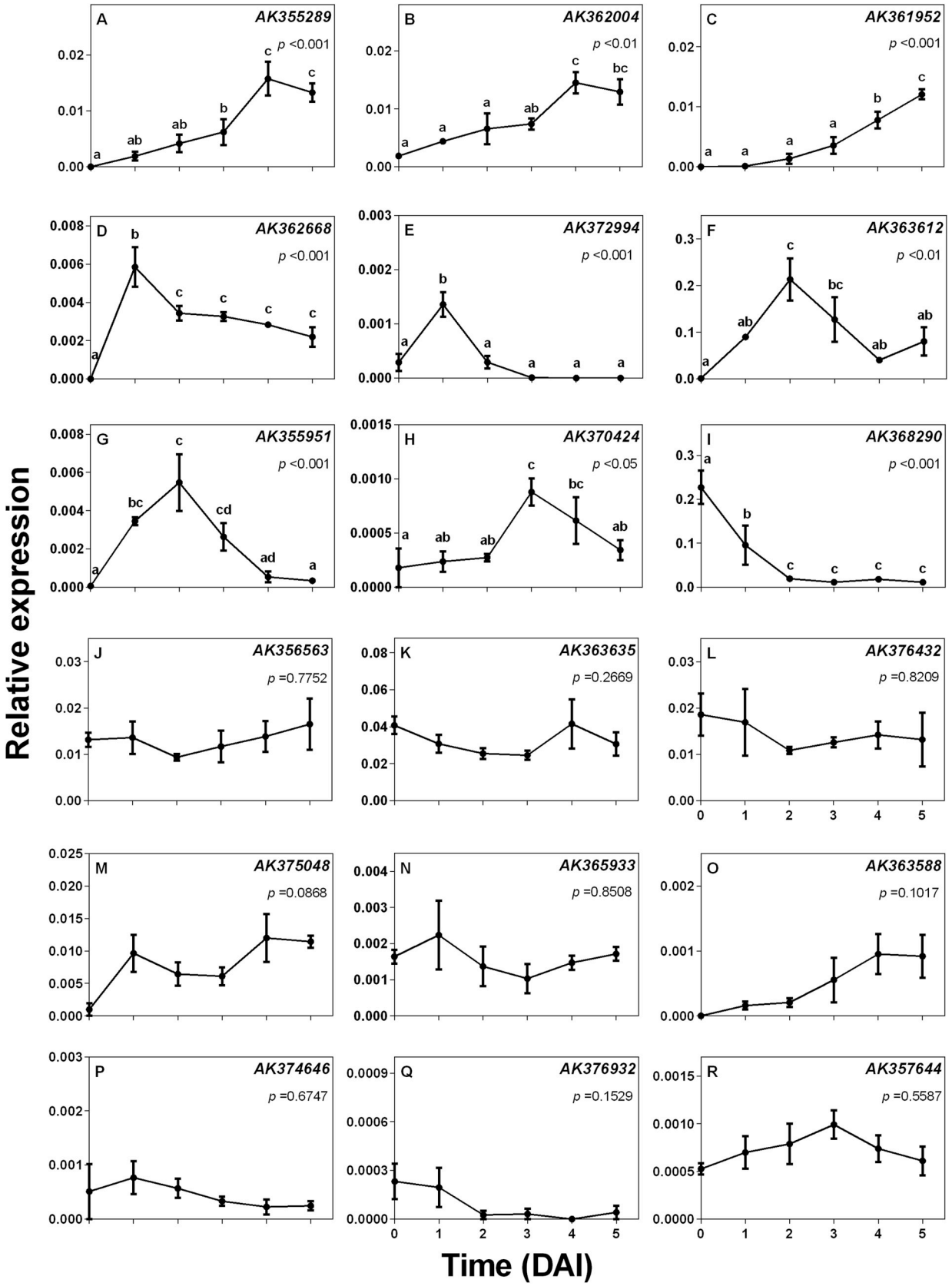

Fig. 7. Relative expression of barley subtilase genes along germination measured by real time PCR. Data represent mean ± SE (n = 3). Different letters show significant differences with *p* ranging from 0.05 to 0.001 as indicated in each graph.

(Roberts et al., 2017) and the remaining 10 were identified by sequence homology in barley databases in the course of the present work. Two of the analyzed subtilase genes, AK355289 (Fig. 7A), AK362004 (Fig. 7B), showed very low expression at the beginning of the germination process but increased with time until 4 DAI reaching an increment of about 6–8 times and remaining at high levels at 5 DAI. Subtilase gene AK361952 (Fig. 7C) showed sustained up-regulation along germination progress with an increment of about 100 times after 5 days. Another five genes showed a pattern of transient induction with a peak at 1 (AK362668, Fig. 7D and AK372994, Figs. 7E) and 2 (AK363612, Fig. 7F and AK355951, Fig. 7G) or 3 (AK370424, Fig. 7H) DAI.

On the other hand, expression of AK368290 (Fig. 7I) was very high at 0 DAI but decreased about 10-fold in the first two days. Of the remaining sequences, ten did not show significant changes along germination regardless expressed at high (AK356563, AK363635 and AK376432) (Fig. 7J to L), low (AK375048, Fig. 7M) or very low (AK365933, AK363588, AK374646, AK376932 and AK357644) (Fig. 7N–R) level, while gene AK376152 was not detected in any sample analyzed.

4. Discussion

4.1. Proteases from different catalytic groups participate in germination associated proteolysis in barley grains

Degradation of storage seed protein during the first stages of germination is essential to sustain early seedling growth. In barley, most articles dealing with protein degradation during germination are restricted to the initial part of the process, typically the first 24–48 h, since that is the period relevant for the malting of grains.

It has been shown that poor or insufficient hydrolysis of storage proteins will prevent solubilisation of starch granules and activity of starch degrading enzymes leading to processing problems, low extract and low fermentation levels (Osman et al., 2002). Excessive proteolysis, otherwise, can affect organoleptic properties of beer, such as flavour, colour and levels of foam-forming proteins (Osman et al., 2002). Therefore, the rate of storage protein degradation during germination can affect the quality of malt and beer. In addition, most of the proteolytic enzymes synthesized in germinating grains remain active in the malt after kilning (Jones et al., 2000; Osman et al., 2002) being able to further contribute to the release of soluble nitrogen during mashing. Therefore, a deeper understanding of the proteolytic mechanism activated during germination and the identification of the enzymes involved together with their native substrates, could provide valuable tools for the brewing industry.

Information about the identity and role of proteolytic enzymes in late stages of germination of barley grains is less abundant. Therefore, in the present work proteolysis was analyzed in germinating seeds from the end of imbibition until advanced seedling growth (up to 5 DAI), when coleoptile and root system were highly developed (Fig. 1A). First significant changes in soluble protein and amino acid concentration are registered within the first 24 h after imbibition while endoprotease activity measured as azocasein hydrolysis starts to increase after that (Fig. 2). The dynamics of soluble protein fraction that increases until day 3 after imbibition and then remains high reflects the simultaneous processes of protein solubilisation and degradation. In agreement, free amino acids accumulate until day 3 and then decrease sharply as being remobilized to the growing tissues (Figs. 1, 2A and 2B). Proteolytic activity increase along germination progress and is maximal between 4 and 5 DAI, when endosperm has been largely modified.

When analyzing the spectrum of proteases involved in germination, a mayor role of cysteine proteases (as determined by E-64 inhibition) was observed (Fig. 3), in accordance with previous reports (Wrobel and Jones, 1992; Jones, 2005). Cysteine proteases EP-A and EP-B, purified from germinating barley grains have been reported as responsible for hordein B and D degradation (Koehler and Ho, 1990). Moreover, transcriptomic studies in barley grains have shown that both proteases are among the most strongly induced genes (> 100 fold increase) during the post-germination phase, defined by the authors as the period between 18 and 71 h of germination (An and Lin, 2011).

Both, chymostatin and TPCK strongly inhibit chymotrypsin-like serine proteases but are also active against some cysteine protease families, such as cathepsins and papain. Most of the reported cysteine proteases active in germinating barley grains belong to these families (Martinez et al., 2009; Diaz-Mendoza et al., 2016) explaining the fact that residual activity measured in the presence of chymostatin and TPCK reached values similar to those obtained with E-64 (Fig. 3).

Contribution by metalloproteases showed a different dynamic to the rest of the catalytic groups, being maximal at 1 DAI (64% of inhibition) and then declining to 44% and 24% of inhibition when using 1,10-phenanthroline or EDTA, respectively, and remaining almost unchanged until the end of the assay. Both, EDTA and 1,10- phenantroline, act as chelators of divalent cations, mainly zinc, iron and calcium, but showing a different inhibitory efficiency in complex mixtures of metalloproteases, as previously reported (Wrobel and Jones, 1992; Zhang and Jones, 1995). Interestingly, in extracts prepared from 3 day germinated barley grains, metalloproteases highly sensitive to 1,10-phenantroline have been shown to remain active even after incubated for 1 h at 90 °C and have been postulated as main responsible for further protein degradation during mashing of kilned malt (Rizvi et al., 2011).

Pepstatin A was used to test for aspartic protease activity. This group accounted for the lowest fraction of azocaseinolytic activity except for day 1 when the activity contributed by aspartic proteases represented about 25% of the total. Previously, activity of aspartic proteases has been quantified by addition of pepstatin A to barley mashes prepared from kilned malt. It was shown that soluble protein decayed when the mashes were incubated in the presence of the inhibitor, but aspartic protease activity using gelatin as substrate remained unchanged. The authors explained that despite the aspartic proteases are important in protein solubilisation, gelatin would not be a good substrate to test the protease activity (Jones and Budde, 2005). Despite our results suggest a minor role for aspartic proteases in germination-associated proteolysis, other authors have demonstrated that several nepenthesin-like aspartic proteinase transcripts increase in abundance during barley germination, suggesting a possible role in protein mobilization (Schmitt et al., 2013).

Concerning serine proteases, a reduction ranging from 10 to 20% of total azocaseinolytic activity was registered when applying PMSF (Fig. 3). Compared to other catalytic groups, activity contributed by serine proteases account for a minor fraction, although significant. Serine proteases varied along germination progress being more active at 1, 3 and 4 DAI. Such fluctuations likely reflect the complexity of the enzymatic group. Moreover, complete inhibition of barley germination was observed when applying PMSF onto intact grains at concentration of 500 μM (Supplemental Figure S1), and a reduction in rootlets and coleoptile growth when applying a lower dose (Fig. 4). Together, these results demonstrate that serine proteases are active players in the germination of barley grains, either in the mobilization of storage protein or other metabolic functions related to germination.

4.2. Subtilases are active players in protein degradation during germination

Subtilases are a family within serine proteases that have been previously associated to the germination process. At least, two serine endoproteases isolated from green malt have been identified as subtilisin-like serine endoproteases (Terp et al., 2000; Fontanini and Jones, 2002). Most studies dealing with protein degradation in germinating barley are restricted to the period relevant to the malting process (Jones, 2005), which in our assay would correspond to samples collected between 1 and 2 DAI. Here, it is shown that subtilase activity increased early after imbibition but also a sustained increment was observed beyond the first stages of grain germination, reaching the

highest values between 4 and 5 DAI (Fig. 5) when rootlets and coleoptile still continue to growth at the expense of grain reserves (Fig. 1). In accordance, SEP-1 activity has been shown to increase up to the sixth day of germination of barley grains when tested against gelatin in 2D native PAGE gels (Fontanini and Jones, 2002). However, neither SEP-1 nor hordolisin were able to hydrolyze isolated hordeins and for this reason a role of these enzymes in reserves mobilization has been previously discarded.

By using a simple and recently developed method (Galotta and Roberts, 2019), subtilase activity was visually revealed on half sections of germinating grains. An intense pink coloration indicating hydrolysis of Suc-AAPF-pNA was visualized in the modified endosperm as germination progressed (Fig. 6). Since endosperm is a dead tissue, all enzymes active during germination must have been synthesized and accumulated during grain development or are produced by the aleurone and embryo during germination. To date, there is no information about the tissue localization of hordolisin in barley grains while SEP-1 activity was detected mainly in the embryo and scutellum and to a lesser extent in the aleurone, but never in the starchy endosperm (Fontanini and Jones, 2002). However, the possibility exists that at least a fraction of one or both of these enzymes actually moves to the endosperm not being detected before. Alternatively, and more likely, other germination associated subtilases not described yet are responsible for the activity here observed.

Interestingly, high subtilase activity was also observed in developing rootlets by our staining method (Supplemental Fig. S2) in accordance with the reported localization of SEP-1 activity in germinating grains (Fontanini and Jones, 2002).

4.3. Subtilase genes up-regulated in germinating barley grains

Eighteen of the 19 subtilase genes analyzed were expressed during the germination of barley grains. Interestingly, eight of them were positively associated to germination progress (Fig. 7A–H). Among the subtilases induced during grain germination, two showed a tendency to increase their gene expression with time reaching significant differences after 3 days and highest expression at 4 DAI (Fig. 7A and B). Both of them remained highly expressed at 5 DAI suggesting that these proteases must fulfill functions in late germination phase when the grains have been largely modified. In a similar way, the sequence AK361952 (Fig. 7C) showed increased expression beyond the first stages of germination involved in malt production and kept increasing until day 5. All these three genes showed high correlation with soluble protein content and Suc-AAPF-pNA hydrolysis measured in soluble extracts of germinating grains (Supplemental Table S2) suggesting that their gene products might be responsible, at least in part, for the subtilase activity observed during germination, together with some other subtilases not identified yet.

Subtilase gene expression has been previously associated to germination in barley by transcriptomic studies. A substantial increment in gene expression of Contig 13847_s_at was observed early after imbibition of barley grains submitted to three different malting conditions ranging from commercial to laboratory scale (Schmitt et al., 2013). Moreover, a positive correlation between Contig 13847_s_at expression and malt extract has been found (Muñoz-Amatriaín et al., 2010). By sequence comparison we found that Contig 13847_s_at corresponds to a fragment of subtilase AK363612 (Fig. 7F) which in our assay showed a peak of gene expression at 2 DAI. Similarly, sequence AK372994 (Fig. 7E) here reported as another subtilase up-regulated early during germination, has been previously recognized as a subtilisin-like serine protease (Contig 20186_at) matching the pattern of protein mobilization in malting experiments (Schmitt et al., 2013).

Gene sequence AK362004 (Fig. 7B) codes for the so far best characterized subtilase in barley. In relation to leaf senescence, it has been reported as one of the two subtilases induced in naturally senescent leaves of barley plants at the vegetative and reproductive phase as well as in dark-induced senescent leaves (Roberts et al., 2017). Closest sequence to AK362004 in wheat codes for serine protease SSP1 purified from detached leaves senescing by incubation in darkness (Wang et al., 2013), that seems to correspond to the previously described subtilase P2 (Roberts et al., 2006) or a closely related protein. Both enzymes, SSP1 and P2, have been independently described as subtilisin-like serine proteases belonging to subfamily S8A and exhibiting high thermo stability as many members of the family. Prediction of subcellular localization of barley subtilase AK362004 suggests that the enzyme is directed to the secretory pathway with final localization in the apoplast (Roberts et al., 2017) although experimental confirmation is still lacking. Here, gene expression of AK362004 was shown to strongly correlate with subtilase activity (Pearson's coefficient = 0.8, $p < 0.001$) and protein degradation (Pearson's coefficient = 0.71, $p < 0.001$) during germination (Supplemental Table S2). Transcriptomic data from studies using the Barley1 Genome Array from Affymetrix available at PLEXdb (http://www.plexdb.org/index.php) reveals that Contig 5955_at and HVSMEi0003D23r2_s_at, both exhibiting highest identity to sequence AK362004, are induced in barley seeds developing under drought stress (Abebe et al., 2010) as well as in aleurone cells of *sln1* barley mutant which expresses constitutive gibberellic acid response in the absence of the hormone (Chen et al., 2010) (Supplemental Table S3). In addition, higher gene expression in the pericarp during early grain development (4–8 days after flowering) and in the embryo of 72 h germinated barley grains have also been measured (Sreenivasulu et al., 2008) in accordance with the increasing gene expression of probe HVSMEi0003D23r2_s_at from stage 2 to stage 5 of germination quantified by other authors (An and Lin, 2011). All this information suggests that the subtilase encoded by gene AK362004 plays an important role during seed development and germination in barley, additionally to the previously reported association to leaf senescence, probably been involved in massive protein degradation programs. Moreover, Plattner et al. (2015) have reported this subtilase as a thionin proprotein-processing enzyme (TPPE), capable of cleaving the acidic domain of the barley leaf-specific thionin BTH6, and releasing the mature protein. While thionin BTH6 has been detected in barley leaves, a distinct group of thionins named hordothionins has been described as specific of barley grain endosperm (Bohlmann et al., 1988). According to Jones and Poulle (1990), a 30 kDa cysteine protease isolated from germinated barley grains is able to hydrolyze the α- and β-hordothionins. It is yet to be evaluated if subtilase AK362004 participates in the processing of grain hordothionins.

The other two sequences encoding putative subtilases, AK355289 (Fig. 7A) and AK361952 (Fig. 7C), and exhibiting high correlation between gene expression, enzymatic activity and protein degradation during germination (Supplemental Table S2) have not been reported before. The highest increment in gene expression for both subtilases takes place in advanced germinated grains suggesting that their function would be more related to late mobilization of reserves needed to sustain the fast growth of seedlings.

A transient induction was observed for another three subtilase genes (Fig. 7D, G and 7H). Interestingly, all of them showed the highest expression at a different time of germination suggesting specific roles for each one of their gene products. Earliest expression peak corresponded to AK362668 at 1 DAI (Fig. 7D) and soon after that, it decayed but maintaining a constant and relatively high expression level along the assay. This subtilase gene has been previously associated to dark-induced senescence in detached barley leaves (Roberts et al., 2017). On the contrary, AK355951 and AK370424 were demonstrated to be down-regulated during dark-induced senescence (Roberts et al., 2017) and in the present work it is shown that both subtilases are transitory induced during germination. Considering that malting of barley grains involved a relatively short germination period compared to the 5 days incubation of our assay, subtilase genes showing early induction, AK362668 and AK355951, appear as the most likely relevant to determination of malt quality together with AK363612 and AK372994, previously reported by

Schmitt et al. (2013).

It is worth to mention that the only subtilase gene down-regulated during germination (Fig. 7I) has also been negatively associated to leaf senescence in young barley plants (Roberts et al., 2017). This gene (sequence AK368290) is highly expressed early after seed imbibition and then it is completely repressed during the first 48 h of germination.

Until today, there is little information about subtilase role/s in germinating seeds and developing seedlings. Studies on seed development seem to be more abundant than those on germination and transcriptomic studies show that subtilases are strongly associated to seed development in many plant species. In Arabidopsis, many subtilases have been reported to be expressed in different tissues of the seed (Supplemental Table S3). In a developmental study of Arabidopsis seeds, several genes encoding subtilases were up-regulated in different seed organs from fertilization to maturity (Belmonte et al., 2013). These subtilases appear to be located in the peripheral endosperm (AT5G58820), the chalazal endosperm (AT4G15040), and the developing embryo suspensor and micropylar endosperm (AT3G46840) (Supplemental Table S3). However, so far there is no information about their biological functions during seed development.

Expression data of two maize subtilases, Zm00001d018281 and Zm00001d020627, obtained with the Genevestigator tool (https://genevestigator.com/gv/) shows that both genes are expressed in roots of developing seedlings, while subtilase encoded by gene Zm00001d022533 is preferentially expressed in the basal endosperm transfer layer of kernels 8 days after pollination (Supplemental Table S3). Interestingly, another maize subtilase, represented by Contig Zm.1284.1.S1_at, is expressed both at seed development and germination (Supplemental Table S3).

An exhaustive analysis of the available information on subtilase gene expression for wheat and rice seeds has been included in a recent publication (Galotta and Roberts, 2019). From those data, we have identified wheat and rice sequences homologous to some of the barley subtilases here described. Wheat subtilase AK448167 shares 93% similarity to barley subtilase AK362668 (Supplemental Figure S3). In wheat, this protease is expressed in germinating seeds, immature inflorescences and developing seeds (Schreiber et al., 2009), while in barley we demonstrated an expression peak early in germination and sustained significant mRNA levels until 6 DAI (Fig, 7D). We have also identified subtilase genes very similar to AK362004 of barley, both in rice (subtilase XM_015770661) and wheat (subtilase JX962746). The rice and wheat sequences share an 81% and 92% of similarity to barley AK362004, respectively (Supplemental Figure S4). They are both expressed in germinating seeds and seedlings in agreement with our results in barley (Fig. 7B). Such a high sequence homology and expression pattern suggests that, at least in cereals, the function of the product of gene AK362004 is conserved among plant species.

5. Conclusions

Subtilases are abundant in nature and fulfill a wide range of biological functions, as in development, protein turnover, defense responses and as components in signaling cascade of many organisms including plants. Both, leaf senescence and seed germination are intrinsically associated to massive protein degradation and nutrient reallocation, and therefore it is not surprising the existence of a pool of proteases playing roles in both processes, more likely related to non-specific proteolysis. The present work contributes to the comprehension of the role of barley subtilases during grain germination and seedling growth by demonstrating the time course increase in subtilase activity and gene expression along the process. This study also allowed the identification of eight subtilase genes positively associated to germination, being five of them reported here for the first time. Further investigations will be required in order to identify their natural substrate (s) and determining their biological functions as well as a possible relevance to malt quality.

CRediT authorship contribution statement

María Florencia Galotta: Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Paulina Pugliese:** Investigation, Methodology. **Flavio H. Gutiérrez-Boem:** Funding acquisition, Writing - review & editing. **Cintia G. Veliz:** Investigation, Methodology. **María Victoria Criado:** Writing - review & editing. **Carla Caputo:** Funding acquisition, Writing - review & editing. **Mariela Echeverria:** Formal analysis. **Irma N. Roberts:** Conceptualization, Investigation, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.021.

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