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Identification and expression analysis of 11 subtilase genes during natural and induced senescence of barley plants

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ABSTRACT

Subtilases are one of the largest groups of the serine protease family and are involved in many aspects of plant development including senescence. In wheat, previous reports demonstrate an active participation of two senescence-induced subtilases, denominated P1 and P2, in nitrogen remobilization during whole plant senescence. The aim of the present study was to examine the participation of subtilases in senescence-associated proteolysis of barley leaves while comparing different senescence types. With this purpose, subtilase enzymatic activity, immunodetection with a heterologous antiserum and gene expression of 11 subtilase sequences identified in barley databases by homology to P1 were analyzed in barley leaves undergoing dark-induced or natural senescence at the vegetative or reproductive growth phase. Results showed that subtilase specific activity as well as two inmunoreactive bands representing putative subtilases increased in barley leaves submitted to natural and dark-induced senescence. Gene expression analysis showed that two of the eleven subtilase genes analyzed, *HvSBT3* and *HvSBT6*, were up-regulated in all the senescence conditions tested while *HvSBT2* was expressed and up-regulated only during dark-induced senescence. On the other hand, *HvSBT1*, *HvSBT4* and *HvSBT7* were down-regulated during senescence and two other subtilase genes (*HvSBT10* and *HvSBT11*) showed no significant changes. The remaining subtilase genes were not detected. Results demonstrate an active participation of subtilases in protein degradation during dark-induced and natural leaf senescence of barley plants both at the vegetative and reproductive stage, and, based on their expression profile, postulate *HvSBT3* and *HvSBT6* as key components of senescence-associated proteolysis.

1. Introduction

Proteases (also referred as peptidases or proteinases) are enzymes able to hydrolyze peptide bonds contained in proteins or peptides of various sizes. They are key components of a wide range of biological processes of great relevance to biology, medicine and biotechnology (Rawlings et al., 2016). In plants, their known biological roles are strikingly diverse, being demonstrated to participate in almost all stages of plant life including meiosis, morphogenesis, embryo development, defense responses, cell death and senescence (Van der Hoorn, 2008; Pesquet, 2012).

Senescence in plants refers to the last phase of development preceding death, either of the whole organism or of at least part of it. Age-related leaf senescence takes place sequentially during the vegetative growth phase allowing successive organ replacement and maintenance of high photosynthesis rates. In addition to single leaf senescence, monocarpic crops also undergo terminal senescence that refers to the whole plant death at the end of the life cycle (Distelfeld et al., 2014). Monocarpic senescence in annual crops is normally induced early in the reproductive phase (Davies and Gan, 2012). Senescence-associated proteolysis plays a crucial role by enabling the remobilization of nutrients, mainly nitrogen (N), from senescent tissues to new developing organs, both during vegetative and reproductive growth phases.

Abbreviations: CBB, Coomassie Brilliant Blue; CPs, cysteine proteases; DAS, days after sowing; N, nitrogen; NSL, non-senescent leaves; RLS, Rubisco large subunit; RSS, Rubisco small subunit; Rubisco, Ribulose-1,5-bisphosphate carboxylase oxygenase (EC 4.1.1.39); SL, senescent leaves; SPs, serine proteases; TCA, trichloroacetic acid; VSL, very senescent leaves.

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Additionally to this nutrient recycling function, senescence-associated proteases are also involved in the regulation of the senescence process. The involvement of proteases in plant senescence is as intricate as reflected by the structural and functional diversity of the proteolytic enzymes that have been associated to this process (Roberts et al., 2012; Díaz-Mendoza et al., 2014). Although our knowledge on senescence-associated plant proteases keeps constantly expanding, for most of them many central questions such as the identity of their natural substrates, biological roles and regulation mechanisms still remain to be answered.

Two catalytic groups have been mainly related to plant senescence, cysteine proteases (CPs) and serine proteases (SPs). CPs, by far the most represented in senescent plant tissues as determined by transcriptomic studies (Bhalerao et al., 2003; Guo et al., 2004; Parrott et al., 2007), have been suggested to participate in leaf protein degradation during senescence in tobacco (Prins et al., 2008; Carrión et al., 2013), wheat (Martínez et al., 2007; Thoenen et al., 2007), barley (Parrott et al., 2010) and oilseed rape (Poret et al., 2016). Specifically, CPs belonging to the papain-like peptidase family C1A, as classified by the MEROPS database (Rawlings et al., 2016), seem to be strongly related to plant senescence development (Díaz-Mendoza et al., 2014). Moreover, the widely used senescence marker *SAG12* which expression strongly correlates with senescence progress in many plant species including barley (see references in Gregersen et al., 2013), encodes an L-like cathepsin belonging to the C1A family.

The role of SPs in senescence have received less attention although they are highly abundant in plants (Tripathi and Sowdhamini, 2006; Van der Hoorn, 2008). According to the MEROPS database plant SPs are divided into 14 families, of which the family S8, known as subtilisin-like proteases or subtilases, is one of the largest. As much as 56 subtilisin-like serine proteases have been identified in the proteome of *Arabidopsis thaliana*, while 63 have been found in rice (Tripathi and Sowdhamini, 2006), all of them belonging to the subfamily S8A. More recently, a total of 80 subtilase genes were identified in the genome of grape (Cao et al., 2014). Despite their abundance, information on the biological role of plant subtilases is still very limited (Schaller et al., 2012) and for many crop species even the number of subtilase genes is unknown. So far, only two wheat peptidases have been classified as subtilases in the MEROPS database, while in barley genome of the 346 counts of known and putative peptidases predicted, only one still unassigned peptidase sequence have been recognized as a member of S8A subfamily (Rawlings et al., 2016).

At present, there is evidence supporting both bulk protein degradation and highly specific regulatory and signaling functions for plant subtilases (Schaller, 2004; Rautengarten et al., 2005; Vartapetian et al., 2011). Subtilases mRNA expression has been shown to substantially increase during senescence in barley (Parrott et al., 2007; Hollmann et al., 2014). Also, increased enzymatic activity at senescence has been reported in different plant species such as wheat (Wang et al., 2013), *A. thaliana* (Martinez et al., 2015), and common bean (Budič et al., 2013). Very recently, the increase in SPs activity measured in leaves of *Brassica napus* senescing in response to N starvation has been attributed in part to four different subtilisins (Poret et al., 2016). In wheat, the purification and characterization of two highly active subtilisin-like proteases from senescent leaves has been previously reported (Roberts et al., 2003, 2006). These proteases were denominated P1 and P2, and it has been suggested that they play an important role in N remobilization during whole plant senescence (Roberts et al., 2011).

Taking into account our previous work in wheat, the aim of the present study was to examine the participation of subtilases in senescence-associated proteolysis in barley. For this purpose, subtilases enzymatic activity was analyzed in dark-induced and naturally senescent leaves of barley plants at the vegetative and reproductive stage. As a complementary approach, immunodetection of putative subtilases in barley leaf protein extracts was attempted by using a heterologous antiserum raised against subtilase P1 from wheat. In addition, barley databases were searched in order to identify sequences coding for putative subtilases related to P1 subtilase from wheat and its mRNA expression profile was examined by qRT-PCR in leaves senescing under dark-induced or natural senescence.

2. Materials and methods

2.1. Plant growth conditions

Studies were conducted in growth chamber or greenhouse. In both cases, barley (*Hordeum vulgare* L.) seeds of the cultivar Scarlett were germinated on filter paper soaked with distilled water for 48 h, before transferring to pots (five seedlings/pot).

2.2. Growth chamber experiments

Plants were grown on vermiculite, watered daily and fertilized every two days with nutrient solution (Hoagland and Arnon, 1950) containing 10 mM KNO_3 and maintained under a photoperiod of 16 h light/8 h dark, at 23 °C and an irradiance of 350 μmol m^{-2} s^{-1}. Fifteen days after sowing (DAS), the last expanded leaf (third leaf) of barley plants was excised (t = 0 d), placed in plastic boxes with distilled water and incubated in complete darkness to induce senescence or maintained under the light/dark cycle used for plant growth as control. Samples of the incubated leaves were taken at 0, 2, 4 and 6 days after detaching. For the analysis of natural senescence in young barley plants, samples of the second, third and fourth leaf were taken starting at 15 DAS and at 21, 25, 28, 32 and 34 DAS. Sampling period took place from full expansion to advanced senescence of the third leaf.

Leaf chlorophyll content was estimated by *in vivo* determination of the greenness index using a SPAD-502 (Konica Minolta Inc., Osaka, Japan). Growth chamber experiments were repeated at least three times. All samples were taken by triplicate.

2.3. Greenhouse experiment

Greenhouse studies were conducted at the School of Agriculture of the University of Buenos Aires in 2013 and repeated in 2014, obtaining similar results. Seedlings were sown in 6-L pots containing a mix of vermiculite, perlite and soil (1:1:2 v/v), fertilized every two weeks with nutrient solution (Hoagland and Arnon, 1950) containing 10 mM KNO_3 and watered periodically to keep substrate at field capacity. Leaf samples were harvested by quadruplicate at 125 DAS and dissected according to age. Plants at this stage, 5.5–5.7 of the Zadoks scale, (Zadoks et al., 1974) had both yellow and green leaves together with developing spikes, indicating a stage of active nutrient remobilization at monocarpic senescence. Three senescence ranks were defined for leaves: non-senescent leaves (NSL, including flag leaf and the last 2 developed leaves), senescent leaves (SL, showing about 40–60% of NSL chlorophyll content) and very senescent leaves (VSL, with less than 10% of NSL chlorophyll content).

Both in growth chamber and greenhouse studies, each replicate consisted of one pot containing five plants. Collected tissues were immediately frozen in liquid N_2 and stored at −80 °C.

2.4. Extracts preparation and biochemical determinations

Frozen leaves were ground with mortar and pestle in liquid N_2 and the powder obtained was extracted with 50 mM Tris-HCl

buffer pH 7.5 and 1% (w/v) polyvinylpolypyrrolidone using 1 mL of buffer per 250 mg of tissue fresh weight (FW). The obtained homogenate was centrifuged at 10,000*g* for 30 min and 4 °C. The supernatant obtained was used for the determination of soluble proteins (Bradford, 1976), Suc-AAPF-pNA (Sigma-Aldrich, St. Louis, MO, USA), hydrolytic activity (Roberts et al., 2006), and source of proteins for SDS-PAGE and immunoblotting as described below. Free amino acids in the supernatant were determined with the ninhydrin reagent (Yemm and Cocking, 1955) after precipitation of proteins with TCA 5% (w/v).

2.5. Polyacrylamide gel electrophoresis and immunoblotting

Aliquots of leaf extracts were fractionated by 10% SDS-PAGE according to Laemmli (1970). Gels were loaded on a leaf FW basis, using equal amounts of each extract (3.75 mg FW) per lane. After electrophoresis, proteins were visualized by Coomassie Brilliant Blue (CBB) R-250 staining or alternatively transferred to PVDF (polyvinylidene difluoride) membranes (Immobilon®, Millipore, Bedford, MA, USA) essentially as described by Towbin et al. (1979). Detection of immunoreactive bands was achieved with a polyclonal antiserum developed in our lab raised against subtilase P1 from wheat (Roberts et al., 2006). Bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG and 1-Step™NBT/BCIP solution (Pierce Biotechnology Inc., Rockford, IL, USA). Images were recorded using the InGenius gel documentation and analysis system (Syngene, Frederick, MD, USA). The integrated density values of the bands of CBB-stained and immunoreactive proteins were determined with Image J software (http://imagej.nih.gov/ij/) from three different gels or blots corresponding to independent replicates, and representative images are shown.

2.6. Identification and structural analysis of barley subtilase genes

Genes coding for putative barley subtilases were identified using the translated BLAST: tblastn tool (Altschul et al., 1990) and wheat P1 partial sequence (APEIFKPDVTAPGVDIL) as query. Multiple sequence protein alignment was constructed using Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and edited manually. Conserved domains in protein sequences were identified using the Conserved Domain Database (CDD) resource at the NCBI web page (http://www.ncbi.nlm.nih.gov/cdd/).

2.7. Total RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from 100 mg of leaf ground tissue using PureLink® Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was checked by spectrophotometry (NanoDrop™, Thermo Scientific, Waltham, MA, USA) and electrophoresis in agarose gels. One microgram of DNase treated RNA was used for cDNA synthesis by reverse transcription using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) and Oligo(dT) following the protocol provided by the manufacturer. cDNA samples were used as templates to analyze the expression of barley subtilases, senescence marker *HvSAG12* (GenBank: AK366134), and two reference genes, actin (GenBank: AY145451) and elongation factor 1-alpha (GenBank: Z50789). Primer sequences are shown in Supplemental Table S1. All primers were designed and tested to have similar Tm values allowing suitable amplification of all genes at the same temperature.

Quantitative PCR analysis (qRT-PCR) was performed in the Stratagene Mx3000P QPCR thermocycler (Agilent Technologies, Santa Clara, CA, USA) with FastStart Universal SYBR Green Master (ROX) from Roche (Productos Roche, Ricardo Rojas, Argentina).

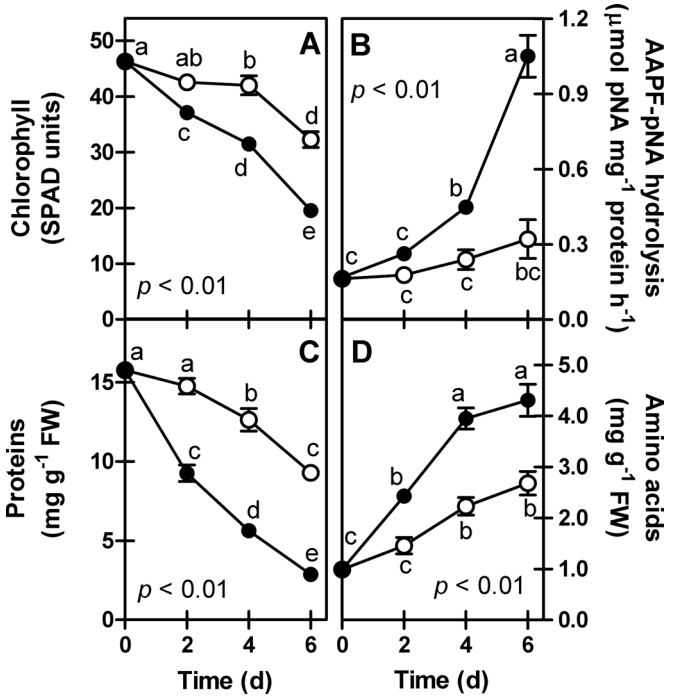


Fig. 1. Changes in chlorophyll (A), suc-AAPF-pNA hydrolytic activity (B), soluble proteins (C) and amino acids (D) in dark-induced senescent leaves of barley. Fifteen days after sowing, the third leaf of each plant was detached and incubated in continuous darkness to induce senescence (closed circles) or maintained under the light/dark cycle used for plant growth (open circles). Samples were taken along time from the moment of detachment (day 0) and until day 6. Data represent means ± SE (n = 3). Different letters show significant differences ($p < 0.01$) obtained by Fisher's LSD test.

Samples were denatured at 95 °C for 10 min, followed by 40 cycles (95 °C for 15 s, 60 °C for 30 s, and 60 °C for 1 min). Comparative Ct (threshold cycles) method (ΔΔCt) was applied for relative quantification of gene expression using the Stratagene Mx3000Pro thermocycler software®.

2.8. Statistical analysis

Analysis of variance and multiple comparison analysis of the data were performed as detailed in each figure legend. Fisher's Least Significant Difference test was used for post-hoc comparisons of means. P-values were considered significant when they were lower than 0.05.

3. Results

3.1. Subtilase activity and immunodetection in dark-induced senescent barley leaves

In order to investigate the relation of subtilases with senescence in barley, enzymatic activity was analyzed in soluble extracts of dark-induced senescent barley leaves by using the chromogenic peptide Suc-AAPF-pNA as a substrate. In these conditions, only 44% of initial chlorophyll content remained after six days of treatment indicating an evident senescence stage (Fig. 1A). Subtilase specific activity significantly increased after four days of treatment and reached a five-fold increase at the end of the assay (Fig. 1B). Concomitantly, soluble protein concentration decreased to less than 20% of the initial content while free amino acids accumulated in the excised leaves (Fig. 1C and D). Detached leaves maintained under the normal light/dark cycle also developed a senescence-type response that was delayed in time, exhibiting significantly

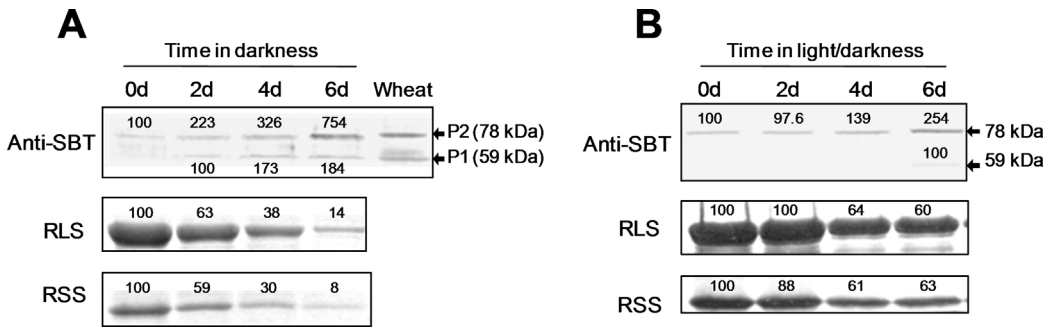


Fig. 2. Rubisco content and immunodetection of putative barley subtilases in detached incubated in continuous darkness (A) or maintained under the light/dark cycle used for plant growth (B). Equal amounts of each extract (3.75 mg FW) were loaded. Leaf proteins were electrophoresed in SDS-PAGE (10%) and Rubisco large (RLS) and small (RSS) subunits were visualized by CBB R-250 staining. To reveal the presence of subtilases, after electrophoresis proteins were transferred to PVDF membranes and subjected to immunoblotting with a polyclonal antiserum raised against wheat subtilases (Anti-SBT). Bands quantification is shown as a percentage of the highest (RLS and RSS) or the first (Anti-SBT) signal detected. Wheat: extract from senescent wheat leaves included to show position of P1 and P2 subtilases.

higher chlorophyll and soluble protein contents by the end of the assay compared to dark-incubated leaves (Fig. 1A and C). At the same time, free amino acid concentration was lower in control leaves (Fig. 1D), while no significant changes in subtilase activity was detected after 6 days of detachment (Fig. 1B).

Next, barley leaf extracts were examined with a polyclonal antiserum raised against P1 subtilase from wheat. Anti-SBT antiserum was previously developed in our lab and has been shown to recognize both P1 and P2 in wheat leaf extracts. Here, when tested against barley leaf extracts, two main immunoreactive bands were revealed, nearly corresponding with the position of the previously described P1 and P2 wheat subtilases (Fig. 2). Analysis of Rubisco large (RLS) and small (RSS) subunit content demonstrated the degree of protein degradation in senescing leaves at the same time that both immunoreactive proteins (putative subtilases) showed increasing amounts (Fig. 2). In agreement with previous observations reported in wheat, a putative barley subtilase corresponding to the lower molecular mass band (named P1 in wheat) was not detectable in non-senescent leaves (Fig. 2A, lane 0d) and became induced after detachment and incubation in darkness (Fig. 2A, lanes 2d to 6d). Concomitantly, the higher molecular mass band resembling wheat protease P2 was already present at day 0 and showed the highest induction (more than 7.5 times) after dark treatment (Fig. 2A). Induction of both immunoreactive bands was also observed in control leaves maintained under the normal photoperiod (Fig. 2B), although to a lesser extent as compared to dark-incubated leaves.

3.2. Identification of subtilase genes in barley databases

Next, in order to identify putative subtilase genes in barley databases, a tBLASTn search was performed by using the previously reported partial sequence of wheat protease P1as a query (APEIFKPDVTAPGVDIL). The most similar sequence in barley corresponded to accession AK365933, containing an exact match to the wheat query. Successive searches, with the newly identified barley gene and its homologous sequences as a query, allowed the recognition of another ten genes coding for putative subtilases. The identified sequences were named *HvSBT1* to *HvSBT11* (Supplemental Table S1). Analysis of the aligned protein sequences revealed identity scores ranging from 35 to 78% (Supplemental Fig. S1). All the identified sequences contained the catalytic triad in the order Asp, His, Ser, characteristic of S8 serine protease family. At least three conserved domains were identified in all the analyzed sequences: PA_subtilisin_like (cd02120), inhibitor_I9 (pfam05922) and peptidases_S8_3 (cd04852), as defined at the Conserved Domain Database (Supplemental Table S2). The 17-amino-acid partial sequence of wheat protease P1was located in

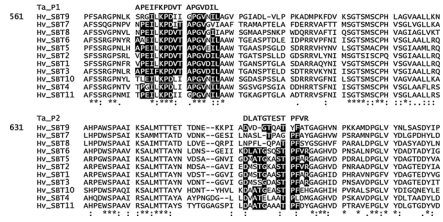


Fig. 3. Partial multiple sequence alignment of barley subtilases showing location of known fragments of P1 and P2 subtilases from wheat. Consensus symbols are used as defined by Clustal Omega tool, (*) indicates fully conserved residues, (:) indicates residues with strongly similar properties and (.) indicates residues exhibiting weakly similar properties. Residues conserved between the two plant species are shaded in black.

a highly conserved region towards the C-terminal end of domain S8-3 while the 13-amino-acid fragment of P2 (DLATGTESTPFVR) was found in a less conserved region (Fig. 3 and Supplemental Fig. S1). Comparison of wheat fragments and barley sequences reveals *HvSBT3* as the most similar to P1 (exact match) and *HvSBT6* the most similar to P2 with 85% identity (Fig. 3).

3.3. Expression analysis of barley subtilases during dark-induced senescence

In order to analyze gene expression of the 11 subtilases during dark-induced senescence, a quantitative real-time-PCR (qRT-PCR) analysis was performed (Fig. 4). Measurement of *HvSAG12* expression as a typical senescence indicator gene allowed the assessment of the extent of the senescence response. *HvSAG12* was up-regulated in leaves showing an advanced senescence stage triggered by darkness, with more than ten times higher expression after six days of incubation (Fig. 4). In these leaves, expression of eight of the 11 subtilase genes analyzed was confirmed. *HvSBT2*, *HvSBT3* and *HvSBT6* showed higher expression in senescent leaves, ranging from 1 to 2 orders of magnitude. On the other hand, *HvSBT1* and *HvSBT7* were strongly repressed in senescent leaves and, even thought not statistically supported, a tendency to lower expression of *HvSBT4* was observed after six days in darkness (Fig. 4). Constant gene expression was recorded for both *HvSBT10* and *HvSBT11* (Fig. 4), while *HvSBT5*, *HvSBT8* and *HvSBT9* were not detected in these leaves.

3.4. Subtilase activity and gene expression in naturally senescing leaves of young barley plants

To better understand the physiological role of each subtilase, the timing of activity and gene expression during the normal

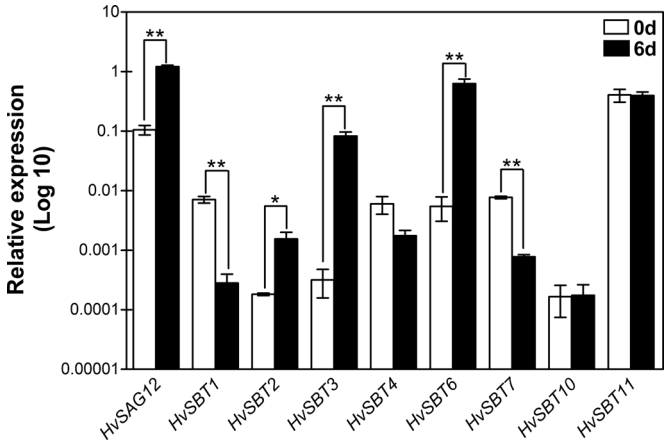


Fig. 4. Relative expression of barley subtilases in dark-induced senescent leaves. Gene expression was analyzed by qRT-PCR in leaves detached from plants at the vegetative stage and incubated in continuous darkness to induce senescence up to six days. Bars represent mean ± SE (n = 3). One asterisk (*) shows significant differences with $p < 0.05$ and two asterisks (**) show significant differences with $p < 0.01$ obtained by Fisher's LSD test when comparing expression values at 0d (white bars) and 6d (black bars) for each gene.

development of senescence in different leaves of plants at the vegetative stage was examined. Senescence, as determined by the decrease in chlorophyll content, started earlier in the second (oldest) leaf, followed by the third and later, the fourth (youngest) leaf (Fig. 5A). The leaf ranks analyzed reached different senescence stages at the end of the assay, with about 10% (leaf 2), 30% (leaf 3) and 68% (leaf 4) of initial chlorophyll content remaining. However, once started, chlorophyll degradation proceeded at very similar rates in leaf 2 and leaf 3.

In the second leaf, induction of subtilase specific activity already began at 28 DAS, reaching almost a 20-fold increase at the last sampling date (Fig. 5B). At the same time, soluble proteins decayed below 10% of the initial amount (Fig. 5C). Meanwhile, subtilase activity in the third leaf started to increase at 32 DAS and reached a peak of 3.5 times initial activity two days later, when about 47% of the protein content had already been hydrolyzed (Fig. 5B and C). Consistent with senescence development and consequent N remobilization, amino acid content decreased in all three leaf ranks (Fig. 5D). In the fourth leaf, even though active protein degradation was observed, no significant changes in subtilase activity were detected (Fig. 5B and C).

In these plants, *HvSAG12* gene expression showed significant increases between the first and last sampling dates in the two oldest leaves, of about 17-fold (leaf 2) and 20-fold (leaf 3) (Fig. 6A). Additionally, *HvSAG12* expression differed with leaf rank at 32 DAS correlating with leaf age. Expression of subtilases *HvSBT3* and *HvSBT6* increased with time in leaf 2 and 3 (Fig. 6C and E) confirming the senescence-associated induction of both genes observed in dark-incubated leaves (Fig. 4). Interestingly, in this experiment *HvSBT2* was neither induced nor detected in any leaf.

On the other hand, *HvSBT1* was negatively associated with senescence according to prior results (Fig. 4), was highly expressed in the fourth leaf at the first sampling date, and then decayed to almost undetectable levels as senescence progressed (Fig. 6B). This gene also showed very low expression both in the second and the third leaves already at day 20 and showed no changes with time.

Similarly, expression of *HvSBT4* was initially higher in young leaf 4 but soon declined with time reaching at 28 DAS very low values comparable to those measured in other leaf ranks (Fig. 6D).

Even though no differences in *HvSBT7* expression were observed among leaves at 21 DAS, an expression peak in the fourth leaf was recorded later (28 DAS), leading to final decrease at 32 DAS. Meanwhile, subtilase *HvSBT7* expression remained constant in the second and third leaves as time progressed (Fig. 6F).

Regarding *HvSBT11* expression, except for a higher initial value in leaf 4 maintained until 28 DAS, no significant changes were measured among leaf ranks or sampling dates (Fig. 6G), as recorded in previous experiment (Fig. 4). Finally, the rest of the subtilase genes under study were not detected in young plants.

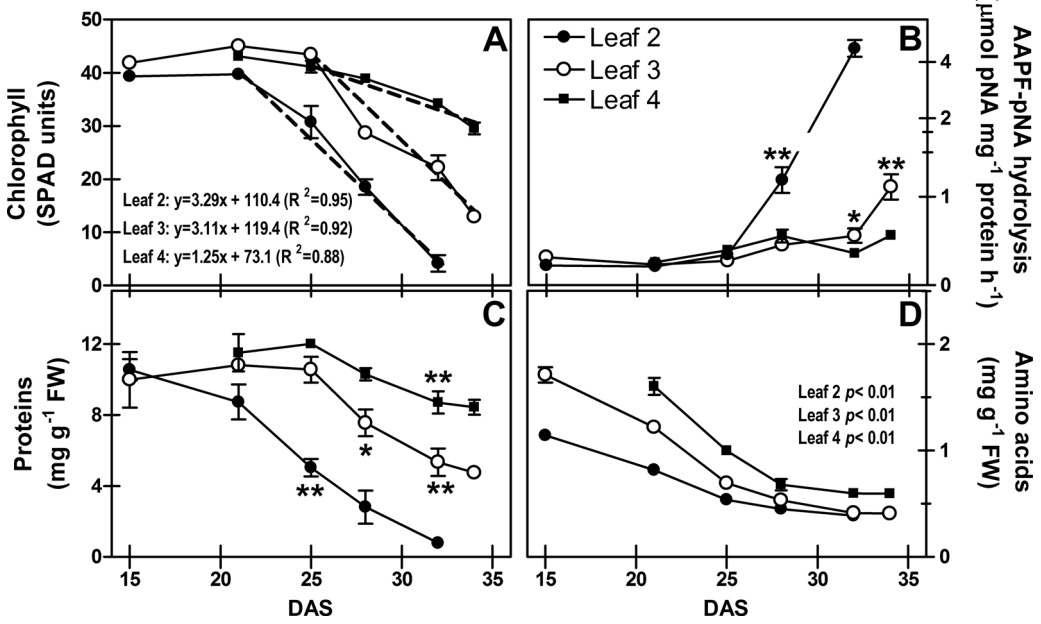
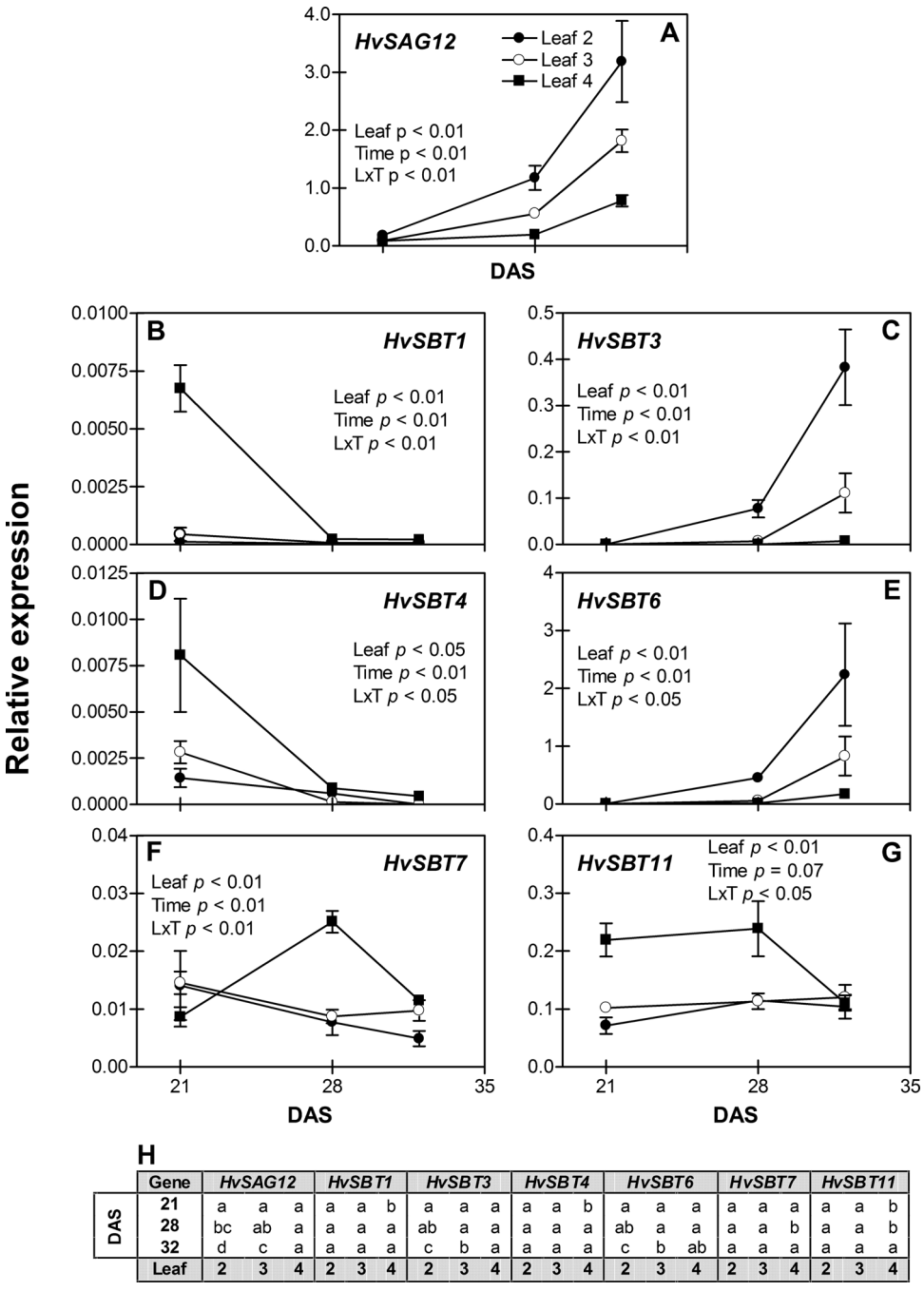


Fig. 5. Changes in chlorophyll (A), suc-AAPF-pNA hydrolytic activity (B), soluble proteins (C) and amino acids (D) in naturally senescing leaves of barley plants at vegetative stage. Samples of the second (black circles), third (white circles) and fourth (black squares) leaf were taken starting at day 15 after sowing and until complete senescence of the third leaf. Data represent means ± SE (n = 3). In all cases, data were analyzed by one-way ANOVA as a function of time for each leaf rank. (A) Linear regression analysis of the data of chlorophyll degradation were performed starting at 22 (leaf 2) and 25 DAS (leaf 3 and 4), shown as dashed lines. (B and C) Asterisks show the first value significantly different from all the previous values of each curve at $p < 0.05$ (*) and $p < 0.01$(**) calculated from one-way ANOVA.



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	Gene	HvSAG12			HvSBT1			HvSBT3			HvSBT4			HvSBT6			HvSBT7			HvSBT11		
DAS	21	a	a	a	a	a	b	a	a	a	a	a	b	a	a	a	a	a	a	a	a	b
	28	bc	ab	a	a	a	a	ab	a	a	a	a	a	ab	a	a	a	a	b	a	a	b
	32	d	c	a	a	a	a	c	b	a	a	a	a	c	b	ab	a	a	a	a	a	a
	Leaf	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4

Fig. 6. Relative expression of barley subtilases in naturally senescing leaves of plants at the vegetative stage determined by qRT-PCR (A-F). Gene expression was analyzed in samples of the second (dark circles), third (white circles) and fourth (dark squares) leaf at 20, 32 and 34 DAS. Data represent means ± SE (n = 3). *P*-values show the level of significance of leaf number, time (T) and their interaction calculated from a two-way ANOVA. Different letters show significant differences obtained by Fisher's LSD test (H).

3.5. Subtilase activity and gene expression in naturally senescing leaves of barley plants at the reproductive phase

Having confirmed the induction of subtilases during natural senescence of leaves from plants at the vegetative growth phase, both enzymatic activity and gene expression were examined in leaves from plants undergoing monocarpic senescence. Therefore, leaf samples of plants at the reproductive stage were harvested and dissected according to age, allowing the definition of three different ranks: non-senescent leaves (NSL), senescent leaves (SL) and very senescent leaves (VSL).

Results showed that in plants in the reproductive phase, subtilase activity also corresponded with the senescence stage of the leaves, with Suc-AAPF-pNA hydrolytic activity being ten times higher in VSL than NSL (Fig. 7B), while lower amounts of chlorophyll (Fig. 7A), soluble proteins (Fig. 7C) and free amino acids (Fig. 7D) indicated a senescence condition correlating with leaf age. However, no significant differences in subtilase activity were observed between NSL and SL, even though both soluble proteins and free amino acids decayed, suggesting the involvement of other senescence-associated proteases different from subtilases (Fig. 7B). The presence of both putative subtilases was revealed by immunorecognition in all leaf ranks collected from plants at the reproductive phase and both exhibited increasing signals according to the senescence stage of the leaves (Fig. 8).

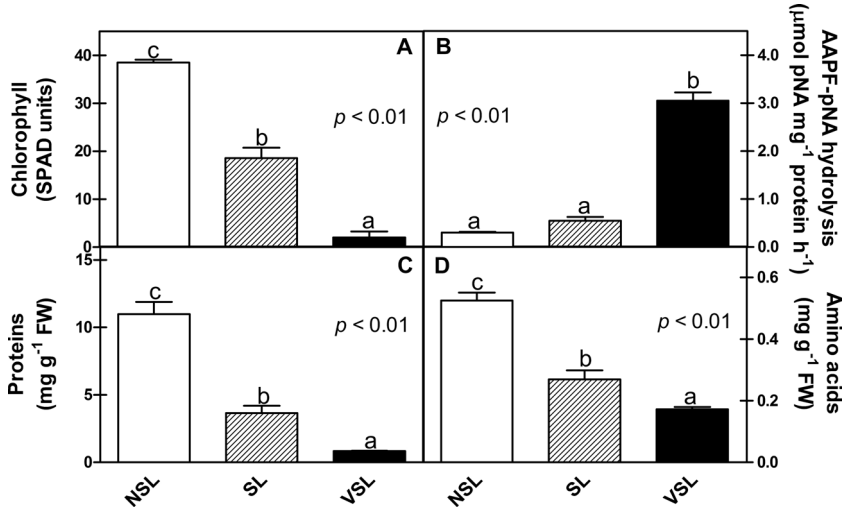

Fig. 7. Changes in chlorophyll (A), suc-AAPF-pNA hydrolytic activity (B), soluble proteins (C) and amino acids (D) in naturally senescing leaves of barley plants at reproductive stage (125 DAS). NSL: non-senescent leaves (white bars), SL: senescent leaves (striped bars), VSL: very senescent leaves (black bars). Data represent means ± SE (n = 4). Different letters show significant differences with p < 0.01 obtained by Fisher's LSD test.

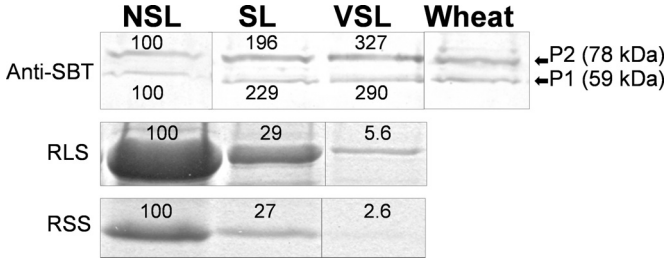

Fig. 8. Rubisco content and immunodetection of putative barley subtilases in naturally senescing leaves of barley plants at reproductive stage. NSL: non-senescent leaves, SL: senescent leaves, VSL: very senescent leaves. Equal amounts of each extract (3.75 mg FW) were loaded. Leaf proteins were electrophoresed in SDS-PAGE (10%) and Rubisco large (RLS) and small (RSS) subunits were visualized by CBB R-250 staining. To reveal the presence of subtilases, after electrophoresis proteins were transferred to PVDF membranes and subjected to immunoblotting with a polyclonal antiserum raised against wheat subtilases (Anti-SBT). Bands quantification is shown as a percentage of the signal detected at 0d. Wheat: extract from senescent wheat leaves included to show position of P1 and P2 subtilases.

In agreement with previous results, *HvSAG12* mRNA expression reflected the senescence status of the different leaves with the highest expression in VSL and the lowest in NSL (Fig. 9A). When subtilase expression was analyzed in these leaves, *HvSBT3* and *HvSBT6* again appeared positively associated with senescence (Fig. 9C and E) as was observed before in leaves from young plants, under natural or dark-induced senescence (Figs. 4, 6C and E). While very low mRNA expression of *HvSBT3* was measured in NLS, an outstanding increase around three orders of magnitude was verified in VSL (Fig. 9C). On the other hand, induction of *HvSBT6* in VSL was about 16 times higher compared to NSL, even though its expression in green leaves was already high compared to *HvSBT3*. No expression of *HvSBT2* was recorded in these leaves as already seen in naturally senescing leaves of young plants (Fig. 6). In agreement with the results obtained with senescent leaves from plants in the vegetative growth phase, *HvSBT1* was strongly repressed in senescent tissues of plants in the reproductive phase (Fig. 9B). On the contrary, no repression of *HvSBT7* was recorded in these leaves (Fig. 9F) as could be expected according to previous data (Figs. 4, and 6F). Finally, *HvSBT4, HvSBT10* and *HvSBT11* did not differ among leaves (Fig. 9D, G, and H) and the rest of the subtilase genes were not detected in any of the samples analyzed.

4. Discussion

4.1. Subtilase activity and relative abundance increase during senescence of barley leaves

Numerous studies addressing senescence characterization in different plant species have been conducted in detached leaves incubated in darkness (Chrost et al., 2004; Talla et al., 2016), the main advantage of this approach being the induction of a fast and synchronized senescence-type response in the incubated leaves. In the present work, this experimental system was selected as a first approach to explore subtilase expression in relation to artificially induced barley leaf senescence. As expected, chlorophyll loss progressed much faster in detached leaves (Fig. 1A) compared to the same leaf in intact plants naturally senescing during vegetative growth (Fig. 5A). Increase of Suc-AAPF-pNAhydrolytic activity under dark-induced senescence of detached leaves has been reported previously in wheat (Roberts et al., 2003), and higher activities have also been observed in leaves of wheat plants senescing under N starvation (unpublished results), highlighting an active role of subtilases in leaf senescence-associated proteolysis triggered by different stress conditions. However, it is very likely that other proteases different from subtilases participate in early senescence-associated proteolysis since first signs of protein degradation seem to precede the highest induction of subtilase activity (Figs. 1, 9C and E).

In addition, in the present work it is shown that subtilase specific activity also correlates with leaf age in plants both in the vegetative (Fig. 5B) and reproductive (Fig. 7B) phases, at the same time as effective amino acid remobilization to sink tissues is taking place (Figs. 5D, 7D). Moreover, this positive association between subtilase activity and tissue senescence stage is not only observed with time progression but also when comparing leaves of different ages along the plant's vertical axis at a particular moment (Figs. 5B, 7B). These results provide evidence in favor of a strong overlap between the responses under artificially induced vs. age-dependent senescence, and in plants in vegetative vs. reproductive phases, at least with regard to the biochemical functions concerning proteolysis and N remobilization.

The role of subtilases in the senescence of barley is also reinforced by the observation that protein bands reacting to heterologous antibodies and most likely corresponding to subtilases were found in extracts from barley leaves submitted to the dark

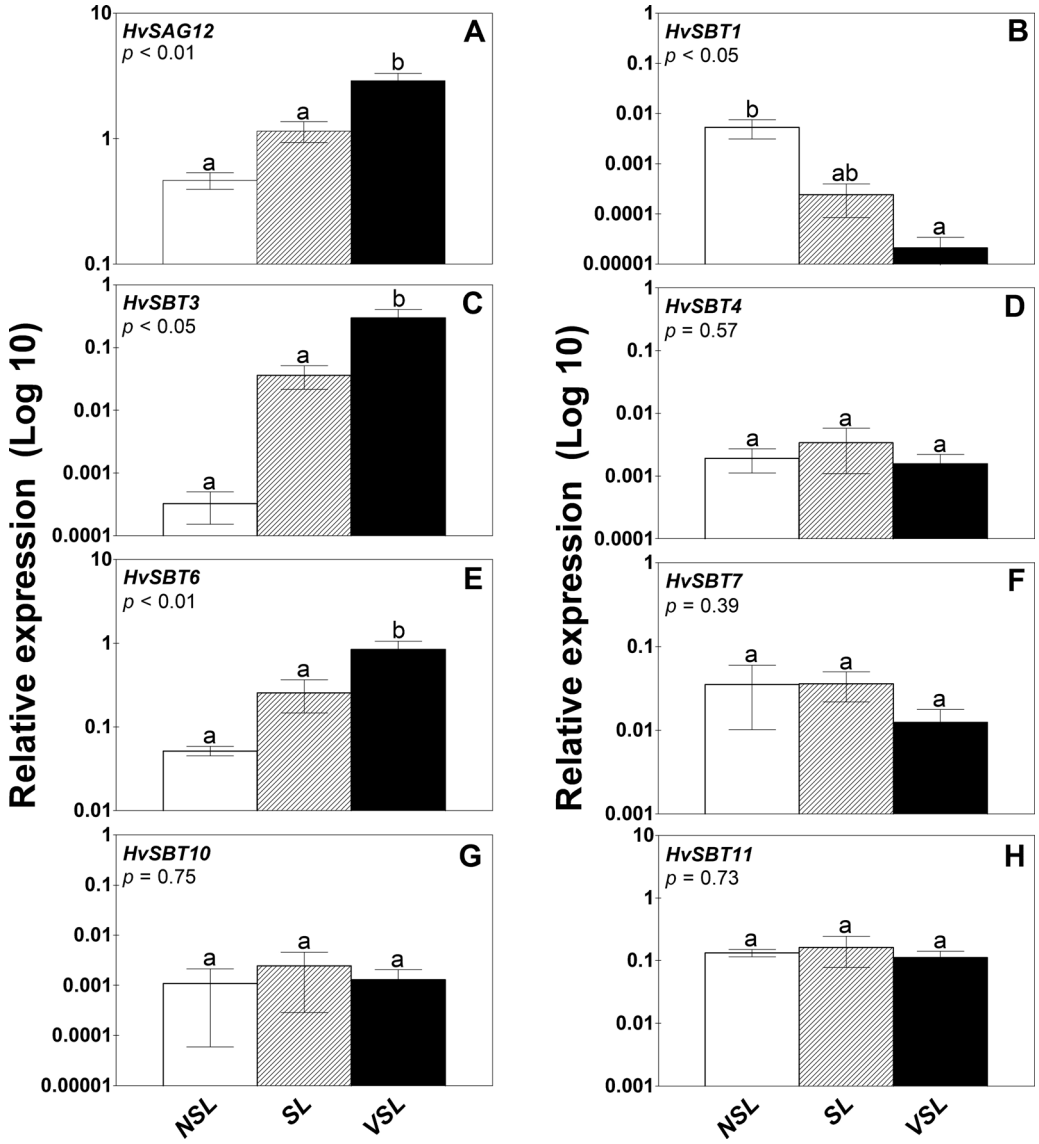

Fig. 9. Relative expression of barley subtilases in leaves of naturally senescing barley plants at reproductive stage determined by qRT-PCR (A–H). NSL: non-senescent leaves (white bars), SL: senescent leaves (striped bars), VSL: very senescent leaves (black bars). Bars represent mean ± SE (n = 4). Different letters show significant differences with $p < 0.05$ (*HvSBT1* and *HvSBT3*) or $p < 0.01$ (*HvSAG12* and *HvSBT6*) obtained by Fisher's LSD test.

treatment (Fig. 2A) and under natural senescence (Fig. 8). The two major protein bands detected strongly resemble molecular mass of P1 and P2 wheat subtilases previously described (Roberts et al., 2006), even though a higher induction of the band resembling P2 in dark-incubated leaves of barley (Fig. 2A) was observed, compared to wheat.

4.2. Subtilase genes induced in senescent barley leaves

Eleven predicted proteins highly similar to the wheat subtilase P1 fragment were identified in barley databases, all of them containing typical subtilase hallmarks in their sequences, essentially the typical catalytic triad of S8 family (Asp, His, Ser) and several conserved domains. A deeper look into barley sequences allowed location of the fragment corresponding to wheat protease P1 in a highly conserved region outside the PA domain but included within the C-terminal end of the peptidase S8 domain. Seven of the 17 wheat residues are fully conserved in all barley subtilases (Fig. 3). Although only *HvSBT3* contains the exact match to the query, identity scores of barley sequences with respect to the wheat P1 fragment ranged from 53% (*HvSBT9*) to 88% (*HvSBT1*, *HvSBT2* and *HvSBT5*). On the other hand, the 13-amino-acid sequence of P2 resides in a less conserved region (Fig. 3), with six of the barley sequences sharing less than 54% identity to P2. The sequence most similar to wheat subtilisin P2 was verified for *HvSBT6* reaching a value of 85% identity, i.e. 11 of the 13 residues fully conserved. Interestingly, barley sequences most closely related to wheat proteases P1 and P2 correspond to *HvSBT3* and *HvSBT6*, respectively, two of the three subtilase genes positively associated with senescence reported here. Both *HvSBT3* and *HvSBT6* showed considerable senescence-dependent induction in all the analyzed conditions, with *HvSBT6* exhibiting higher expression values in all tissues and *HvSBT3* being the most strongly induced (Figs. 4, 6 and 9). However, since to date no other information about P1 and P2 genes is available, except for the fragments sequenced from purified proteins, it is not possible to establish an explicit correspondence between them and barley subtilases *HvSBT3* and *HvSBT6*. Nonetheless, the evidence indicates that the relation of these genes and their products with plant senescence deserves further research, as it does in the case of *HvSBT2*, the other subtilase gene showing

senescence-dependent induction even though it was only detected in the dark treatment (Fig. 4).

Intensive research has been conducted in order to establish the similarities and differences among natural or artificially induced senescence at the physiological, morphological and molecular levels (Weaver and Amasino, 2001; Springer et al., 2015). Although typical senescence symptoms such as chlorophyll and Rubisco degradation are shared by natural and artificially induced senescence systems, substantial differences in the expression pattern of particular genes have been demonstrated (Becker and Apel, 1993; Park et al., 1998; Buchanan-Wollaston et al., 2005) suggesting the existence of alternative pathways for essential metabolic processes operating under different senescence types (Buchanan-Wollaston et al., 2005). Therefore, the possibility exists that different subsets of proteases are responsible for bulk leaf protein degradation depending on the senescence inductor (age, hormones, biotic or abiotic stresses). This seems to be the case for subtilase *HvSBT2*, which shows enhanced gene expression only under dark-induced senescence (Fig. 4), evidencing a clear difference from *HvSBT3* and *HvSBT6*, which also up-regulated under age-dependent senescence (Figs. 6 and 9).

Interestingly, both *HvSBT2* and *HvSBT6* have been cloned initially as cDNAs expressed in barley seedlings after treatment with abscisic acid, a critical plant hormone involved in the regulation of a broad range of abiotic and biotic stress responses together with many physiological processes during normal plant growth and development, including senescence (Finkelstein, 2013). In addition, *HvSBT3* expression seems to be related not only to dark-induced and normal leaf senescence but also to other stress conditions, since it has been found in barley seedlings under salt treatment and abscission (Matsumoto et al., 2011) (Supplemental Table S3).

Previous reports demonstrating up-regulation of subtilase genes in barley include a global study of gene expression in leaves senescing under high C/N ratios imposed by steam-girdling (Parrott et al., 2007). Carbohydrate accumulation in leaves, as a consequence of phloem interruption, leads to a senescence acceleration response characterized by the up-regulation or down-regulation of more than 100 protease genes. The authors reported the at least two-fold induction of three subtilase genes, among which the contig 12029_s_at, identified as a subtilisin-like serine protease, showed the highest increase in expression. Similarly, induction of a subtilase gene (accession TA42918_4513) has been described in senescing flag leaves of field grown barley both under standard and high N supply (Hollmann et al., 2014). Both sequences show highest identity (E-value = 0) to accession AK365933 of *H. vulgare*, here named *HvSBT3*. Additionally, primer sets used to confirm subtilase expression by qPCR in the respective aforementioned articles show a 100% match with accession AK365933, demonstrating that *HvSBT3* and the subtilases reported by Parrott et al. (2007) and Hollmann et al. (2014) are the same sequence.

In the Affymetrix Barley 1_22k microarray, only two probes show high identity to the *HvSBT2* cDNA sequence (Supplemental Table S4), one of them corresponding to contig12029_s_at, which we have established as specific for *HvSBT3*. The other one, probe EBpi01_SQ004_E18_at, apparently is more specific for *HvSBT2* (100% identity in a fragment of 105 nucleotides) and has been shown to be up-regulated in young leaves from barley plants after 14 days of iron deficiency (Higuchi et al., 2011). In the same work, up-regulation of contig12029_s_at was also observed in young leaves after 25 days under iron deficiency, supporting the idea that this probe represents a gene different from *HvSBT2*. Additionally, microarray data demonstrates that probes associated with *HvSBT3* (contig12029_s_at and contig12030_at) are up-regulated in developing barley caryopses under drought stress (Mangelsen et al., 2011). Interestingly, all the existing data on gene expression of probes related to *HvSBT3* suggest a role in different stress responses besides its strong association with leaf senescence reported here and supported by the work of Christiansen and Gregersen (2014) using the Agilent Barley 4×44 microarray. These authors have demonstrated the induction of two subtilases in senescing flag leaves of barley plants, one of them corresponding to *HvSBT3* (accession TA42919_4513, which shows 100% identity to contig12029_s_at) and the other one (accession TA41537_4513) related to rice subtilase RSP1 (Yamagata et al., 2000).

HvSBT6 is represented by two probes with high identity and coverage: contig5955_at (99% over 478 nucleotides) and HVSMEi0003D23r2_s_at (95.7% over 300 nucleotides) (Supplemental Table S4), both subjected to up-regulation in barley seeds developing under drought stress (Mangelsen et al., 2011).

An updated Blast search using *HvSBT2* and *HvSBT3* cDNA sequences as queries revealed that most similar sequences in *A. thaliana* are accessions NM_126136 (16% cover, 68% identity, e-value 4×10^{-24}) and NM_112261 (14% cover, 68% identity, e-value 2×10^{-21}), respectively (Supplemental Table S5). NM_126136 is referred to as ARA12 subtilase protein (AtSBT1.7, At5g67360), while NM_112261 is one of the accessions corresponding to the subtilase recently denominated as SASP (AtSBT1.4, At3g14067) (Martinez et al., 2015). Both Arabidopsis subtilases have been associated with specific biological functions. ARA12 was first reported to localize mainly in stems and siliques (Hamilton et al., 2003) and later in the developing seed coat, and is necessary for normal mucilage released during germination (Rautengarten et al., 2008). Available information on SASP describes its localization in the vacuole (Carter et al., 2004) as well as in the apoplastic fluid of Arabidopsis rosettes (Boudart et al., 2005). In addition, a role in the regulation of branching and silique development has been assigned to this protease (Martinez et al., 2015). However, even when the identity between Arabidopsis and barley sequences is relatively high, in both cases the query sequence coverage is too short to presume similar biological functions in both plant species. Actually, SASP gene sequence is more closely related to *HvSBT6* (52% query cover, 68% identity, e-value 3×10^{-75}) (Supplemental Table S5).

The analysis of barley sequences in relation to other grasses shows that highly similar sequences are found in foxtail millet, maize, sorghum and *Brachypodium distachyon*, but all of them correspond to uncharacterized predicted proteins. The only exception is for *HvSBT6* with the wheat sequence JX962746 as best hit (99% query cover, 92% identity, e-value 0.0), corresponding to the senescence-associated subtilisin protease SSP1(Wang et al., 2013). SSP1 was purified from senescent wheat leaves induced by incubation in darkness for 48 h and its biochemical properties seem very similar to our subtilase P2 (Roberts et al., 2006) except for the differences in enzyme sensitivity to specific inhibitors and molecular weight of the purified protein, which can be easily attributed to different methodological approaches used to characterized both proteases. A molecular weight of 98.9 kDa was determined for SSP1 by natural gradient PAGE (Wang et al., 2013), while a molecular weight of 78 kDa was reported for P2 by SDS-PAGE (Roberts et al., 2006), this discrepancy possibly being related to the conformational protein state (native vs. denatured). Similarly, other minor differences in sensitivity of the proteases to different inhibitors or pH range can be explained by the selection of different methods to assay enzymatic activity (in-gel gelatine degradation vs. hydrolysis of AAPF-pNA in solution). Thus, it seems likely not only that SSP1 and P2 are the same protein but also that they correspond to the gene product of the wheat gene orthologous to barley gene *HvSBT6*.

Subcellular localization prediction reveals that barley subtilases are mainly directed to the secretory pathway (Supplemental Table S3), as are numerous plant subtilases characterized so far (Schaller et al., 2012; Cao et al., 2014). However, our results consistently show that both *HvSBT3* and *HvSBT6* in barley, and P1 and P2 subtilases in wheat (Roberts et al., 2006, 2011), are induced under

natural and stress-triggered senescence. For this reason, a potential contribution of subtilases in N remobilization deserves further research as well as confirmation of their genuine subcellular localization.

4.3. Subtilase genes repressed in senescent barley leaves

Three of the 11 analyzed subtilase genes, *HvSBT1*, *HvSBT4* and *HvSBT7*, were expressed in non-senescent (green) leaves and showed a negative association with senescence progress in barley leaves, in at least some of the assayed conditions. Most consistent expression data showing senescence-associated down-regulation correspond to *HvSBT1*, the expression of which consistently decayed under induced and natural senescence both in vegetative and reproductive phases (Figs. 5, 6 and 9). On the other hand, *HvSBT7* expression seems to develop a different behavior depending on leaf age; while no significant changes were detected in leaves 2 and 3 along the senescence process, a transient induction in leaf 4 between 21 and 28 DAS preceded a sharp fall to initial expression values at 32 DAS (Fig. 6F). This suggests that *HvSBT7* might be restricted to very specific role/s during the short period soon after complete leaf expansion and prior to senescence onset.

A negative association of *HvSBT1* and *HvSBT7* with senescence does not look unreasonable, since both have been reported to be expressed in seedlings submitted to abiotic stresses such as low temperature and aluminium toxicity, respectively (Supplemental Table S3). In addition, *HvSBT1* has been cloned from barley seedlings treated with jasmonic acid.

Even though no significant changes in *HvSBT4* expression were measured by qRT-PCR either in leaves submitted to the dark treatment (Fig. 4) or in leaves from mature plants (Fig. 9D), a negative correspondence with senescence was verified in leaves from young plants both in an age-dependent way at 21 DAS and over time for leaf 4 (Fig. 6D). These results suggest a different expression profile of *HvSBT4* in young versus mature plants (i.e. vegetative senescence versus monocarpic senescence). So far, known physiological conditions for *HvSBT4* expression are limited to barley seedlings under salinity stress and abscission or aluminium toxicity (Supplemental Table S3).

4.4. Non senescence-related subtilase genes

The rest of the subtilase genes analyzed in this work did not show an apparent relation with leaf senescence, since no significant changes were determined (*HvSBT10* and *HvSBT11*) or moreover, no expression at all was detected (*HvSBT5*, *HvSBT8* and *HvSBT9*) in the different assays performed.

In the case of *HvSBT10*, originally cloned from germinated shoots (Supplemental Table S3), even though very low and erratic measurements were recorded, a reliable quantification was possible in samples of the dark-treated (Fig. 4) and mature plants (Fig. 9G). Because no significant changes were seen in either experiment, a main role of this subtilase in N remobilization can be presumably discarded. Similarly, since there is no information on the source tissue from which *HvSBT11* was identified (Supplemental Table S3), and considering that only a slight decrease of about one half in gene expression was observed in leaf 4 of young vegetative plants (Fig. 6G), no assumptions about its function in senescence can be made so far, even when the relatively high expression level of this gene in all the physiological conditions tested is worth noting.

Finally, three of the 11 subtilase genes analyzed were not detected under any experimental condition tested here. Considering that *HvSBT8* and *HvSBT9* were originally cloned from early flowers (Supplemental Table S3), the lack of signal of these genes in barley leaves may indicate a tissue-specific expression pattern. On the other hand, *HvSBT5* identified from a cDNA library constructed from seedling shoots would be reflecting differences in gene expression according to plant developmental stage.

Interestingly, *HvSBT8* lacks the TT motif at the end of the inhibitor domain (Supplemental Fig. S1), thought to be needed for the autocatalytic processing of subtilase zymogens (Meyer et al., 2016). Thus it can be presumed that even when it undergoes efficient transcription, the resulting translation product would be functionless.

5. Conclusions

To date, available information on the role of subtilases in plant senescence remains very limited, including in the particular case of barley. In this work, we explored the potential participation of subtilases in senescence-associated proteolysis of barley leaves. All the parameters here analyzed (enzymatic activity, protein amount and gene expression) demonstrate the involvement of some members of the subtilase family in senescence development. Two of the 11 subtilase genes analyzed, *HvSBT3* and *HvSBT6*, were up-regulated in all the senescence conditions tested, suggesting an involvement in proteolytic functions central to the senescence process. Meanwhile, *HvSBT2* was up-regulated only during the senescence-type response triggered by detachment and dark-incubation of the leaves, likely reflecting a role in more specific stress responses. However, since diverse microarray studies have revealed variable expression patterns for these genes under different stresses or developmental phases (e.g. germination), further studies are necessary to establish the precise biological functions of the identified subtilases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.01.005.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Becker, W., Apel, K., 1993. Differences in gene expression between natural and artificially induced leaf senescence. Planta 189, 74–79.
Bhalerao, R., Keskitalo, J., Sterky, F., Erlandsson, R., Björkbacka, H., Birve, S.J., Karlsson, J., Gardeström, P., Gustafsson, P., Lundeberg, J., Jansson, S., 2003. Gene expression in autumn leaves. Plant. Physiol. 131, 430–442.
Boudart, G., Jamet, E., Rossignol, M., Lafitte, C., Borderies, G., Jauneau, A., Esquerré-Tugayé, M.T., Pont-Lezica, R., 2005. Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: identification by mass spectrometry and bioinformatics. Proteomics 5, 212–221.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P.O., Nam, H.G., Lin, J.F., Wu, S.H., Swidzinski, J., Ishizaki, K., 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J. 42, 567–585.
Budič, M., Sabotič, J., Meglič, V., Kos, J., Kidrič, M., 2013. Characterization of two novel subtilases from common bean (*Phaseolus vulgaris* L.) and their responses to drought. Plant Physiol. Biochem. 62, 79–87.
Cao, J., Han, X., Zhang, T., Yang, Y., Huang, J., Hu, X., 2014. Genome-wide and molecular evolution analysis of the subtilase gene family in *Vitis vinifera*. BMC Genomics 15, 1–15.
Carrión, C.A., Costa, M.L., Martínez, D.E., Mohr, C., Humbeck, K., Guiamet, J.J., 2013. In vivo inhibition of cysteine proteases provides evidence for the involvement

of 'senescence-associated vacuoles' in chloroplast protein degradation during dark-induced senescence of tobacco leaves. J. Exp. Bot. 64, 4967–4980.
Carter, C., Pan, S., Zouhar, J., Avila, E.L., Girke, T., Raikhel, N.V., 2004. The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell 16, 3285–3303.
Christiansen, M.W., Gregersen, P.L., 2014. Members of the barley NAC transcription factor gene family show differential co-regulation with senescence-associated genes during senescence of flag leaves. J Exp. Bot. 65, 4009–4022.
Chrost, B., Daniel, A., Krupinska, K., 2004. Regulation of α-galactosidase gene expression in primary foliage leaves of barley (*Hordeum vulgare* L.) during dark-induced senescence. Planta 218, 886–889.
Díaz-Mendoza, M., Velasco-Arroyo, B., González-Melendi, P., Martínez, M., Díaz, I., 2014. C1A cysteine protease–cystatin interactions in leaf senescence. J. Exp. Bot. 65, 3825–3833.
Davies, P.J., Gan, S., 2012. Towards an integrated view of monocarpic plant senescence. Russ. J. Plant Physiol. 59, 467–478.
Distelfeld, A., Avni, R., Fischer, A.M., 2014. Senescence, nutrient remobilization, and yield in wheat and barley. J. Exp. Bot. 65, 3783–3798.
Finkelstein, R., 2013. Abscisic acid synthesis and response. Arabidopsis Book 11, e0166.
Gregersen, P.L., Culetic, A., Boschian, L., Krupinska, K., 2013. Plant senescence and crop productivity. Plant Mol. Biol. 82, 603–622.
Guo, Y., Cai, Z., Gan, S., 2004. Transcriptome of Arabidopsis leaf senescence. Plant Cell Environ. 27, 521–549.
Hamilton, J.M., Simpson, D.J., Hyman, S.C., Ndimba, B.K., Slabas, A.R., 2003. Ara12 subtilisin-like protease from Arabidopsis thaliana: purification, substrate specificity and tissue localization. Biochem. J. 370, 57–67.
Higuchi, K., Saito, A., Mikami, Y., Miwa, E., 2011. Modulation of macronutrient metabolism in barley leaves under iron-deficient condition. Soil Sci. Plant Nutr. 57, 233–247.
Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stat. Circ. 347, 1–39.
Hollmann, J., Gregersen, P.L., Krupinska, K., 2014. Identification of predominant genes involved in regulation and execution of senescence-associated nitrogen remobilization in flag leaves of field grown barley. J. Exp. Bot. 65, 3963–3973.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Mangelsen, E., Kilian, J., Harter, K., Jansson, C., Wanke, D., Sundberg, E., 2011. Transcriptome analysis of high-temperature stress in developing barley caryopses: early stress responses and effects on storage compound biosynthesis. Mol. Plant 4, 97–115.
Martínez, D.E., Bartoli, C.G., Grbic, V., Guiamet, J.J., 2007. Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. J. Exp. Bot. 58, 1099–1107.
Martinez, D.E., Borniego, M.L., Battchikova, N., Aro, E.-M., Tyystjärvi, E., Guiamét, J.J., 2015. SASP, a Senescence-Associated Subtilisin Protease, is involved in reproductive development and determination of silique number in Arabidopsis. J. Exp. Bot. 66, 161–174.
Matsumoto, T., Tanaka, T., Sakai, H., Amano, N., Kanamori, H., Kurita, K., Kikuta, A., Kamiya, K., Yamamoto, M., Ikawa, H., 2011. Comprehensive sequence analysis of 24,783 barley full-length cDNAs derived from 12 clone libraries. Plant Physiol. 156, 20–28.
Meyer, M., Leptihn, S., Welz, M., Schaller, A., 2016. Functional characterization of propeptides in plant subtilases as intramolecular chaperones and inhibitors of the mature protease. J. Biol. Chem. 291, 19449–19461.
Park, J.-H., Oh, S.A., Kim, Y.H., Woo, H.R., Nam, H.G., 1998. Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. Plant Mol. Biol. 37, 445–454.
Parrott, D.L., McInnerney, K., Feller, U., Fischer, A.M., 2007. Steam-girdling of barley (*Hordeum vulgare*) leaves leads to carbohydrate accumulation and accelerated leaf senescence, facilitating transcriptomic analysis of senescence-associated genes. New Phytol. 176, 56–69.
Parrott, D.L., Martin, J.M., Fischer, A.M., 2010. Analysis of barley (*Hordeum vulgare*) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. New Phytol. 187, 313–331.
Pesquet, E., 2012. Plant proteases—from detection to function. Physiol. Plant. 145, 1–4.
Poret, M., Chandrasekar, B., van der Hoorn, R.A.L., Avice, J.-C., 2016. Characterization of senescence-associated protease activities involved in the efficient protein remobilization during leaf senescence of winter oilseed rape. Plant Sci. 246, 139–153.
Prins, A., van Heerden, P.D.R., Olmos, E., Kunert, K.J., Foyer, C.H., 2008. Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. J. Exp. Bot. 59, 1935–1950.
Rautengarten, C., Steinhauser, D., Büssis, D., Stintzi, A., Schaller, A., Kopka, J., Altmann, T., 2005. Inferring hypotheses on functional relationships of genes: analysis of the Arabidopsis thaliana Subtilase gene family. PLoS Comp. Biol. 1, e40.
Rautengarten, C., Usadel, B., Neumetzler, L., Hartmann, J., Büssis, D., Altmann, T., 2008. A subtilisin-like serine protease essential for mucilage release from Arabidopsis seed coats. Plant J. 54, 466–480.
Rawlings, N.D., Barrett, A.J., Finn, R., 2016. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 44, D343–D350.
Roberts, I.N., Murray, P.F., Caputo, C.P., Passeron, S., Barneix, A.J., 2003. Purification and characterization of a subtilisin-like serine protease induced during the senescence of wheat leaves. Physiol. Plant. 118, 483–490.
Roberts, I.N., Passeron, S., Barneix, A.J., 2006. The two main endoproteases present in dark-induced senescent wheat leaves are distinct subtilisin-like proteases. Planta 224, 1437–1447.
Roberts, I.N., Caputo, C., Kade, M., Criado, M.V., Barneix, A.J., 2011. Subtilisin-like serine proteases involved in N remobilization during grain filling in wheat. Acta Physiol. Plant. 33, 1997–2001.
Roberts, I.N., Caputo, C., Criado, M.V., Funk, C., 2012. Senescence-associated proteases in plants. Physiol. Plant. 145, 130–139.
Schaller, A., Stintzi, A., Graff, L., 2012. Subtilases-versatile tools for protein turnover, plant development, and interactions with the environment. Physiol. Plant. 145, 52–66.
Schaller, A., 2004. A cut above the rest: the regulatory function of plant proteases. Planta 220, 183–197.
Springer, A., Acker, G., Bartsch, S., Bauerschmitt, H., Reinbothe, S., Reinbothe, C., 2015. Differences in gene expression between natural and artificially induced leaf senescence in barley. J. Plant Physiol. 176, 180–191.
Talla, S.K., Panigrahy, M., Kappara, S., Nirosha, P., Neelamraju, S., Ramanan, R., 2016. Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. J. Exp. Bot. 67, 1839–1851.
Thoenen, M., Herrmann, B., Feller, U., 2007. Senescence in wheat leaves: is a cysteine endopeptidase involved in the degradation of the large subunit of Rubisco? Acta Physiol. Plant. 29, 339–350.
Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.
Tripathi, L.P., Sowdhamini, R., 2006. Cross genome comparisons of serine proteases in Arabidopsis and rice. BMC Genomics 7, 1.
Van der Hoorn, R.A., 2008. Plant proteases: from phenotypes to molecular mechanisms. Ann. Rev. Plant Biol. 59, 191–223.
Vartapetian, A.B., Tuzhikov, A.I., Chichkova, N.V., Taliansky, M., Wolpert, T.J., 2011. A plant alternative to animal caspases: subtilisin-like proteases. Cell Death Differ. 18, 1289–1297.
Wang, R., Liu, S., Wang, J., Dong, Q., Xu, L., Rui, Q., 2013. Purification, characterization and identification of a senescence related serine protease in dark-induced senescent wheat leaves. Phytochemistry 95, 118–126.
Weaver, L.M., Amasino, R.M., 2001. Senescence is induced in individually darkened Arabidopsis leaves, but inhibited in whole darkened plants. Plant Physiol. 127, 876–886.
Yamagata, H., Uesugi, M., Saka, K., Iwasaki, T., Aizono, Y., 2000. Molecular cloning and characterization of a cDNA and a gene for subtilisin-like serine proteases from rice (*Oryza sativa* L.) and *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem. 64, 1947–1957.
Yemm, E., Cocking, E., 1955. The determination of amino-acids with ninhydrin. Analyst 80, 209–213.
Zadoks, J.C., Chang, T.T., Konzak, C.F., 1974. A decimal code for the growth stages of cereals. Weed Res. 14, 415–421.