Regulation of senescence-associated protease genes by sulphur availability according to barley (*Hordeum vulgare* L.) phenological stage

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- **Background and Aims** Proteases are responsible for protein degradation during leaf senescence, allowing nutrients to be redirected to sink tissues. In a previous work, we reported that sulphur deficiency produced a delay in the leaf senescence of barley (*Hordeum vulgare* L.) plants, both at vegetative and reproductive stages. In this work we analyse the effect of sulphur deficiency on the expression of several genes coding for proteases of different catalytic groups, which have been strongly associated with leaf senescence.
- **Methods** Four independent experiments were performed in order to impose low sulphur availability conditions: one of steady-state sulphur deficiency during vegetative stage and three of sulphur-starvation during vegetative and reproductive stages.
- **Key Results** Sulphur deficiency inhibited or reduced the senescence associated induction of seven of the eight proteases analysed. Their induction, as well as senescence and phloem amino acid remobilization, could be achieved with senescence inductors such as methyl-jasmonate (a hormonal stimulus) and darkness, but with different rates of induction dependent on each gene. Sulphur deficiency also exerted an opposite effect on the gene expression of two cysteine-proteases (*HvSAG12* and *HvLEGU*) as well as on one serine-protease (*HvSUBT*) according to leaf age and plant phenological stages. All three genes were induced in green leaves but repressed in senescent ones of sulphur deficient plants at the vegetative stage. At the reproductive stage both cysteine-proteases were only repressed in senescent leaves while the serine-protease was induced in green and senescent leaves by sulphur deficiency.

- **Conclusions** Our results highlight the relevance of adequate sulphur nutrition in order to ensure leaf senescence onset and proteases' genes induction, which will consequently impact on grain protein composition and quality. In addition our results provide evidence that leaf age, plant developmental stage and the nature of the stress modulate the sulphur responses.

Key words: barley (*Hordeum vulgare* L.), gene expression, proteases, senescence, sulphur deficiency**.**

INTRODUCTION

In cereals such as barley (*Hordeum vulgare* L.), up to 90% of the nitrogen (N) stored in leaves during vegetative growth can be mobilized to the grains in the form of amino acids when senescence takes place (Gregersen *et al.*, 2008). Leaf senescence is basically a process of nutrient recycling where there is a massive degradation of complex molecules, such as proteins, and a redirection of those nutrients towards tissues under development, which leads to the death of the senescing leaves and organs (Buchanan-Wollaston, 1997). Senescence can occur throughout the life cycle of a plant during the vegetative growth, with a translocation of nutrients to the younger plant's parts, and during the reproductive stage of monocarpic plants, where all tissues and organs of the parental plant die to remobilize the nutrients from the senescing tissues to the developing seeds (Distelfeld *et al.*, 2014).

In general, senescence onset of both organs (as leaf) and whole plant (as monocarpic) is induced by abiotic stresses such as N deficiency (Avice and Etienne, 2014; Poret *et al.*, 2019). However, it has been reported that sulphur (S) deficiency can delay leaf senescence in oilseed rape at the vegetative stage (Dubousset *et al.*, 2009; Abdallah *et al.*, 2011) and in barley leaves both at the vegetative and reproductive stages (Veliz *et al.*, 2014, 2017). Until a few decades ago, S availability in agricultural soils was not a major issue for crop production. However, deficiency of this nutrient has arisen as an important limitation for crops in several regions of the world. The decrease in S availability is a consequence of a lack of nutrient replacement that are exported with crops together with soil erosion (Lavado and Taboada, 2009). Also, the environmental policies applied in Europe to reduce atmospheric S depositions by industries and the use of purer nitrogenous and phosphorus fertilizers that no longer contain traces of S contribute to S deficiency in soils (Schnug and Haneklaus, 1998; Scherer, 2001; Haneklaus *et al.*, 2007). This deficiency affects not only yield but also quality of crops (Haneklaus *et al.*, 2007).

During leaf senescence protein degradation is mainly carried out by proteases (Buchanan-Wollaston *et al.*, 2003; Kant *et al.*, 2011; Avice and Etienne, 2014) which are detected in all different cellular compartments including special vesicles like senescence-associated vacuoles (SAVs), rubisco-containing bodies (RCBs) and chloroplast vesicles (CV). Those vesicles are of importance not only in proteolysis and carbon and N remobilization during senescence but also during biotic and abiotic stress conditions (Diaz-Mendoza *et al.*, 2016; Otegui, 2018; Izumi *et al.*, 2019; Zhuang and Jiang, 2019).

Proteases are a large superfamily of hydrolytic enzymes that can also fulfil a myriad of other key functions at all stages of the plant life cycle. The turnover of proteins, the degradation of damaged, misfolded or potentially harmful proteins, the selective breakdown of growth regulatory proteins, post-translational modifications, the maturation of enzymes, development and defence functions (Schaller, 2004; van der Hoorn, 2008; Roberts *et al.*, 2012) are some of them.

The most used protease classification is based on their active site residues, divided mainly in cysteine-, serine-, aspartic-, and metallo-proteases (Grudkowska and Zagdańska, 2004; van der Hoorn, 2008). Their biological functions and roles are varied and may overlap between families (van der Hoorn, 2008). Cysteine-proteases are the most represented in plant senescent tissues (Roberts *et al.*, 2012) and can rise to 90% of the total proteolytic activity, involving protein maturation, degradation, protein rebuilding in response to different external stimuli and playing a house-keeping function to remove abnormal/misfolded proteins (Grudkowska and Zagdańska, 2004). Serine-proteases are the largest class of proteases in plants, involving both bulk protein degradation and highly specific regulatory and signalling functions. Besides, they play a crucial role in N remobilization, especially during monocarpic senescence (Schaller, 2004; Roberts *et al.*, 2012). Aspartic-proteases may have a role in plant defence, processing of seed storage proteins, and in protein degradation for the mobilization

of N during seed germination (Schaller, 2004; Roberts *et al.*, 2012). Finally, metallo-proteases involve a divalent cation for their activity, and have a degradative or highly specific processing function (Schaller, 2004), depending on the member analysed, although there are few reports about their participation in senescence (Roberts *et al.*, 2012).

In order to better understand the effect of S deficiency on leaf senescence, here the expression of eight protease genes was analysed under different S availabilities in green and senescent leaves, as well as at vegetative and reproductive stages. The genes were selected for being previously associated with leaf senescence (Eason *et al.*, 2005; Hara-Nishimura *et al.*, 2005; Parrott *et al.*, 2007, 2010; Roberts *et al.*, 2012) and for representing four different catalytic groups of proteases. They are the cysteine-proteases papain (*HvPAP*), senescence-associated gene 12 (*HvSAG12*), aleurain (*HvALEU*) and legumain (*HvLEGU*); the serine-proteases subtilisin (*HvSUBT*) and carboxi-peptidase III (*HvCPMIII*); the aspartic-protease chloroplast nucleoid DNA-binding protein 41 (*HvCND41*); and the metallo-protease leucine-aminopeptidase (*HvLEU-AP)*. Also, the protease's expression was studied under artificial senescence induction by methyl-jasmonate (MJ) in darkness. Both, the phytohormone and darkness treatment, are well-known to markedly accelerate leaf senescence when applied exogenously (Ueda and Kato, 1980; Weidhase *et al.*, 1987; Parthier, 1990; Smart, 1994; Quirino *et al.*, 2000; Weaver and Amasino, 2001).

MATERIALS AND METHODS

Plant material and experimental design

For experiments 1 – 3, barley plants (*Hordeum vulgare* L. cv. Scarlett) were grown in 300 cm^3 pots with vermiculite as substrate in a growth chamber at 16 h photoperiod and an irradiance of 350 □mol m^{-2} s^{-1}, with temperatures of 24/18°C day/night and a relative humidity of 65%. For experiment 4, barley plants were grown in 6 L pots with

vermiculite/perlite (1:1) as substrate in a greenhouse at the School of Agriculture of the University of Buenos Aires (Argentina, 34º59'S, 58º48'W) with natural light and temperature from June to November in 2014 and 2016. In all cases, each sampling unit consisted of one pot containing five plants. Each experiment was repeated twice, giving similar results between them.

Plants were watered periodically with nutrient solution containing 1 mM K_2SO_4, 0.2 mM $MgSO_4.7H_2O$, 1 mM $CaCl_2.2H_2O$, 0.2 mM KH_2PO_4, 10 mM KNO_3, 0.08% (w/v) Na_2EDTA, 0.03% (w/v) $FeCl_3.6H_2O$, 3 □M H_3BO_3, 0.03 □M $Na_2MoO_4.2H_2O$, 0.75 □M $ZnSO_4.7H_2O$, 0.1 □M $MnSO_4.1H_2O$, 0.2 □M $CuSO_4.5H_2O$ and 0.2 □M $CoSO_4.7H_2O$. Standard nutrient solution containing 1.2 mM $S\text{-}SO_4^{-2}$ was defined as the high S (HS) control treatment for all experiments. Low S (LS) treatment contained 1.25 □M of $S\text{-}SO_4^{-2}$ contributed only by micronutrient salts since K_2SO_4 and $MgSO_4.7H_2O$ were eliminated from the nutrient solution. In this case, potassium and magnesium were provided as 1 mM KCl and 0.2 mM $MgCl_2.6H_2O$.

Experiment 1 - S starvation during vegetative stage Plants were watered with HS nutrient solution until the third leaf emerged (ten days after sowing, DAS). Then, plants were divided into two groups: control and S-starved plants. Control plants were maintained with the same growing solution while S-starved plants were changed to LS solution. Samples of the source leaf (third leaf) were collected at 21 and 38 DAS, corresponding to full leaf expansion and complete leaf senescence in control plants, respectively. Fresh samples were weighed, frozen in liquid N_2 and stored at -80ºC for further biochemical and molecular analysis. For plant biomass determination (as dry weight, DW), shoots from another group of plants were collected and dried at 60°C until constant weight. Leaf stage was determined visually (Supplementary data photographs) and by greenness index (GI) monitoring measured *in vivo* by using a SPAD (Soil Plant Analysis Development) analyser (Konica-*Minolta*, Japan). Five

ranks of senescing leaves were defined from the complete expanded leaf GI of each treatment as: green (GR, more than 80% GI), early senescence (ES, between 60 and 80% GI), middle senescence (MS, between 40 and 60% GI), advance senescence (AS, less than 40% GI) and dead (D, when no GI could be recorded).

Experiment 2 - Senescence induction in S-starved plants The experiment was conducted as described above, but the source leaf of each plant was collected at 26 DAS, when the third leaf of control plants was in ES stage. At that moment detached leaves were incubated in darkness with 100 □M MJ up to three days and then were collected at 0, 2 and 3 days after MJ + darkness application (days after incubation, DAI), weighed, frozen in liquid N_2 and stored at -80ºC. DW of the whole plant was determined before leaf detachment at 0 DAI and leaf stage was determined as mentioned above.

Experiment 3 - Steady-state S deficiency The LS and HS treatments were established from the beginning of the experiment. At 16, 28 and 35 DAS, samples of the source leaf (second leaf) were collected and processed as mentioned for experiment 1.

Experiment 4 - S starvation during reproductive stage Plants were watered with HS nutrient solution until 51 DAS (when the first tillers appeared, Z1.3, Z2.1 according to Zadoks scale); and then the S starvation treatment was applied. Samples of the source leaves (the two leaves below the flag leaf) were collected during grain filling at 106 (anthesis for both treatments), 120, 129 and 135 DAS and processed as mentioned for the other experiments.

Phloem exudates

Phloem exudates were obtained from leaves of experiment 1 at 21 and 38 DAS and of experiment 2 after each MJ + darkness incubation period by the EDTA-facilitated method, as described by Caputo and Barneix (1997). They were incubated in 20 mM EDTA (pH 8) for 3 h at 25ºC in the dark to obtain an exudation solution. The exudation solution was stored at -

20°C for further phloem amino acid determination by the ninhydrin method (Yemm and Cocking, 1955).

Biochemical analysis

Plant material was ground with liquid N_2 in a mortar until a fine powder. Ground tissue (250 mg of fresh weight, FW) were homogenized in 1 ml of 25 mM Tris-HCl buffer (pH 7.5) and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10,000 *g* and 4ºC for 30 min. After centrifugation, supernatant was recovered and used for colorimetric determination of soluble proteins (Bradford, 1976) and free amino acids (Yemm and Cocking, 1955). For measurement of free leaf amino acids, a protein removal step with 5% (v/v) trichloroacetic acid and centrifugation at 10,000 *g* for 10 min was performed.

Total RNA Extraction, cDNA Synthesis, and quantitative PCR

Total RNA was extracted from 100 mg of ground leaves with TRIzol® Reagent (*Ambion*, USA) following the manufacturer's protocol. RNA quality was checked with a NanoDrop™ (*Thermo Scientific*, USA) and by electrophoresis in agarose gels. Total RNA (1 □g) treated with DNAse was used for cDNA synthesis by reverse transcription with *M-MLV* reverse transcriptase (*Promega*, USA) and oligodt, following the manufacturer's protocol. The cDNA aliquots were used as templates to quantify the expression of the genes of interest. Reverse transcription (RT) qPCR was carried out with the Stratagene *Mx3000Pro QPCR* thermocycler (*Agilent Technologies*, USA) by using *FastStart Universal SYBR Green Master (ROX)* from *Roche* (Argentina). Samples were denatured at 95°C for 10 min, followed by 40 cycles (95°C for 15 s, 58-62°C for 30 s, and 60°C for 1 min) and 1 cycle of dissociation (95ºC for 1 min, 55ºC for 30 s and 95ºC for 30 s). Proteases and reference genes (*HvActin* and *HvEF1*□) analysed, together with their primer sequences are detailed in Table 1. For relative

quantification of gene expression, comparative threshold cycle method (□□Ct) was applied using the software provided with the *Stratagene Mx3000Pro* thermocycler.

Statistical analysis

Analysis of variance (*ANOVA*) of S, time (T) and their interactions were performed. A Fisher's Least Significant Difference (LSD) test was used for post-hoc comparisons of means. In graphics and tables different letters were added when a significant TxS interaction was found in order to visualise the differences in S response in time. *P*-values were considered significant when lower than 0.05. Four and three independent replicates were analysed for biochemical and molecular analysis, respectively.

RESULTS

Experiment 1 – S starvation during vegetative stage

After 11 days of S starvation (DAT) leaf developmental stage, total aerial biomass (DW) and leaf chlorophyll content (GI) were similar for both S treatments (Table 2). At 28 DAT S-starved plants showed less signs of senescence, higher chlorophyll content and lower DW than control plants (Table 2). Soluble protein content fell with time in both treatments, with a tendency to show lower values in S-starved plants while leaf amino acid content remained constant in control plants and fell in S-starved plants (Table 2).

While no effect under S starvation was observed at 11 DAT on protease genes expression, at 28 DAT a lower expression of all the genes analysed was observed in S-starved plants with respect to control ones (Fig. 1). Except for *HvCND41* (Fig. 1G), all the proteases showed an increasing expression with time in control plants (Fig. 1). In S-starved plants, only *HvSAG12*, *HvALEU* and *HvLEGU* increased with time, although to a lesser extent compared to control plants (Fig. 1B - D); *HvPAP* showed a strong tendency to increase (Fig. 1A; SxT $p = 0.08$); *HvSUBT*, *HvCPMIII* and *HvLEU-AP* remained constant (Fig. 1E, F and H) and

HvCND41 decayed with time, as observed in control plants, but with a lesser expression level (Fig. 1G).

Experiment 2 – Senescence induction in S-starved plants

In order to analyse if the senescence delay together with the lack of protease induction observed in S-starved plants in experiment 1 could be overcome, leaf senescence was triggered by MJ and darkness application.

Before MJ + darkness treatment, source leaf chlorophyll content was higher in S-starved compared to control leaf, in accordance with the establishment of a GR and ES stage, respectively. Besides, at that moment S-starvation had already impacted negatively over the DW, soluble proteins and free amino acids, which were lower in S-starved than in control plants (Table 2; 0 DAI). Then, as incubation time progressed, chlorophyll content decreased in control as well as in S-starved plants, at a similar rate since no TxS interaction was found (Table 2). At 3 DAI, the control leaf was completely dead, while S-starved leaves showed a chlorophyll content equivalent to that of the control leaf at 2 DAI (Table 2). Protein content also decreased and amino acid content increased as incubation time progressed, but at a lower rate in S-starved plants compared with control ones (Table 2).

Both at 0 and 2 DAI, all the proteases analysed showed a lower expression in the S-starved plants compared to the control ones (Fig. 2), except for *HvCND41* (Fig. 2G) and *HvALEU* at 2 DAI (Fig. 2C). Besides, *HvPAP*, *HvSAG12*, *HvLEGU* and *HvCDN41* showed a similar rate of induction or repression for both treatments (Fig. 2A, B, D, G), while *HvALEU*, *HvSUBT*, *HvCPMIII* and *HvLEU-AP* presented a differential effect of S in time (Fig. 2C, E, F, H). *HvALEU* remained constant at a high level in control plants while increased in S-starved ones (Fig. 2C). *HvSUBT*, *HvCPMIII* and *HvLEU-AP*, instead, increased at a lesser rate in S-starved than in control plants (Fig. 2E, F, H). Therefore, the final expression of

HvPAP, *HvSAG12* and *HvLEGU* (similar rate) reached the maximum value obtained in the control plants one day later, that of *HvALEU* (higher rate) surpassed it while that of *HvLEU-AP* (lower rate) did not reach it (Fig. 2; Table 3). However, even though the rate of induction of *HvSUBT* and *HvCPMIII* were lower in S-starved than in control ones, they reached to a similar and higher level of expression respectively at 3 DAI (Fig. 2; Table 3).

Experiment 3 – Steady-state S deficiency

Under continuous S deficiency (LS), total aerial biomass, chlorophyll and protein content were lower while leaf amino acid content was higher at 16 DAS with respect to well S supplied (HS) plants (Table 2). At that moment, only *HvPAP* gene expression was negatively affected by S-deficiency (Fig. 3A). But interestingly, a higher expression level of *HvSAG12* (Fig. 3B), *HvLEGU* (Fig. 3D) and *HvSUBT* (Fig. 3E) were recorded in LS respect to HS plants. For the rest of the genes analysed, no significant effects were observed (Fig. 3). In order to evaluate whether the aforementioned inductions could also be observed at the senescence stage, the assay was continued until complete leaf senescence of HS (28 DAS) and LS (35 DAS) plants. Again, DW increment and chlorophyll decay in time was significantly lower in LS than HS with a positive TxS interaction that evidence an exacerbation of the S deficiency effect over time (Table 2). Also, as it was observed in experiment 1 the three genes reached lower values in LS plants, both at 28 and at 35 DAS compared to HS plants (Table 4). In this way, a reduction in the rate of induction in time was established (Table 4).

Experiment 4 – S starvation during reproductive stage

The aerial biomass increased in both treatments with time, but the S-starved plants showed a lower DW at all the times analysed (Table 2). The chlorophyll content decreased to a lower rate in S-starved compared to control leaves which recorded a lower value at 55 and 69 DAT, but higher at 84 DAT than control plants (Table 2).

Soluble protein content in source leaves fell with time equally in both treatments, although S-starved plants presented a lower protein content than control ones all along the assay (Table 2). Leaf amino acid content remained constant in control plants throughout the experiment (Table 2), and although no S-starvation effect was observed, a fall between 69 and 78 DAT in S-starved plants was recorded (one-way *ANOVA* T $p = 0.02$).

HvSAG12 and *HvLEGU* showed the same expression level in green leaves for both treatments and then reached lower values in S-starved compared to control plants in senescent leaves (Fig. 4A, B). However, *HvLEGU* showed an earlier induction in time in the S-starved compared to control plants (at 78 DAT; Fig. 4B). *HvSUBT*, instead, showed a similar expression pattern in time in both treatments but with a higher expression level in the S-starved compared to control plants both in green and senescent leaves (Fig. 4C).

Experiment 1 and 2 – Phloem amino acid remobilization

In the S starvation assay at 11 DAT the rate of phloem amino acid remobilization was similar for both S treatments but at 28 DAT, when control plants showed an AS leaf stage (Table 2), control leaves presented a higher phloem amino acids export rate than S-starved ones (Fig. 5A).

In the senescence induction assay at 0 DAI there was no significant effect between S treatments. At 2 DAI there was an induction of the phloem amino acid remobilization for both treatments, but this induction was lower in the S-starved compared to the control plants. At 3

DAI the S-starved plants reached similar phloem amino acids values than those obtained for the control plants at 2 DAI (Fig. 5B).

DISCUSSION

To our knowledge this is the first report of S deficiency impact over the expression pattern of different protease genes previously associated with leaf senescence (Eason *et al.*, 2005; Hara-Nishimura *et al.*, 2005; Parrott et al., 2007, 2010; Roberts *et al.*, 2012), belonging to all the principal catalytic groups. As proteases carry out protein degradation of senescing leaves to give free amino acids to supply grain nutrient demand (Buchanan-Wollaston *et al.*, 2003; Kant *et al.*, 2011; Avice and Etienne, 2014), their alteration in leaves will consequently impact on grain composition and quality. It is key to remark that N remobilization efficiency during the vegetative stage (named as sequential leaf senescence) has a marked impact in the agronomic goal of high seed yield and quality (Avice and Etienne, 2014), therefore the study of its regulation is of key importance.

The results obtained in control leaves corroborated the senescence-associated induction of seven of the eight proteases under study in barley leaves at vegetative stage (Fig. 1A - F and H). *HvCND41*, instead, showed a down-regulation with time (Fig. 1G) as it has been previously reported in naturally senescing leaves of barley during grain filling (Parrott *et al.*, 2007; Jukanti and Fischer, 2008). This suggests that *HvCND41* may not be associated with natural (or MJ+darkness-induced) senescence progress neither at reproductive nor vegetative stage in barley leaves. However, this aspartic protease has been positively

associated with senescence induction by carbohydrate accumulation after leaf girdling in barley (Parrott *et al.*, 2007, 2010), as well as in senescence of other species such as Arabidopsis (Diaz *et al.*, 2008) and tobacco (Nakano *et al.*, 2003; Kato *et al.*, 2004, 2005).

On the other hand, under S-deficiency the senescence-associated induction of the protease genes was lower than that observed in the control plants both during natural (Figs. 1B – D; 4A, B) and induced senescence (Fig. 2A – F and H) and even in some cases there was no induction (Fig. 1A, E, F and H). These results, together with the slower decrease in time of the chlorophyll content of the source leaves of S-starved plants and the lack of free amino acid accumulation in senescent leaves (Table 2) confirm the delay in the senescence progress due to S deficiency reported previously in barley (Veliz *et al.*, 2014, 2017) and oilseed rape (Dubousset *et al.*, 2009; Abdallah *et al.*, 2011).

With the application of MJ and darkness, the expression of most of the proteases in the S-starved plants reached similar levels to that of the control ones, although delayed in time (Fig. 2; Table 3). Such induction was accompanied by a fall in chlorophyll and soluble protein content as well as the accumulation of free amino acids in the source leaf (Table 2), all characteristics of the progression of leaf senescence (Avice and Etienne, 2014). Therefore, these results confirmed that the restriction in S availability causes a delay in the progression of the senescence program but does not prevent it.

Interestingly, MJ + darkness treatment greatly induced the phloem amino acid remobilization of both control and S-starved plants (Fig. 5B), although an inhibition of this parameter is established under S deficiency (Veliz *et al.*, 2014, 2017; Fig. 5A). These results suggest that the senescence and proteolysis delay in S-deficient plants may be related with the inhibition of remobilization by a reduction in protein-derived amino acids available for exportation, which in turn could be the consequence of the visualization of the S-stress

symptoms (i.e. chlorosis, growth retardation, inhibition of protein synthesis, among others) in younger tissues (Schnug and Haneklaus, 1998).

Besides, here we provide evidence that S deficiency differentially altered protease gene expression pattern according to leaf age and plant phenological stages. At the vegetative stage S deficiency not only inhibits protease induction in senescent leaves but also induces several genes (*HvSAG12*, *HvLEGU* and *HvSUBT*) in green leaves, although for this to occur an acclimation period and/or a minimum restriction time of S is required, since it was observed only in the steady-state assay after 16 DAT (Fig. 3 B, D, E) but not in the S-starved assay after 11 DAT. On the other hand, in the reproductive stage S deficiency generated an induction of *HvSUBT* independently of the leaf age (green or senescent, Fig. 4C), while it did not exert any affect over *HvSAG12* and *HvLEGU* expressions in green leaves but generated a lower expression level of both genes in the senescent leaves (Fig. 4A, B) in agreement with the results observed for experiment 1 (Fig. 1B, D).

Therefore, the results obtained here show that the shortage of S and the senescence induction does not exert the same effect on all the proteases analysed, even within the same catalytic group, highlighting that senescence-associated gene expression is governed by a complex transcriptional regulatory network dependent on multiple factors in addition to the simple senescence triggering (Li *et al.*, 2012; Zhang and Gan, 2012), such as the stage of the leaf, the whole plant development, the nature of the stress and hormone application.

Besides, the induction of *HvSAG12*, *HvLEGU* and *HvSUBT* by S deficiency suggests that these enzymes may fulfil different biological functions throughout the life cycle of the plant, in addition to those associated with massive protein hydrolysis during senescence (Lohman *et al.*, 1994; Weaver *et al.*, 1998; Roberts *et al.*, 2011; Avice and Etienne 2014; Roberts *et al.*, 2017), such as those related to the turnover of proteins for the recycling of S (Hesse *et al.*, 2004; Nikiforova *et al.*, 2004), as well as the removal of abnormal or non-

functional proteins (Grudkowska and Zagdańska, 2004; Schaller, 2004), since S has a key role in the formation and maintenance of protein structure and functionality (Haneklaus *et al.*, 2007). In fact, S limitation is associated with an oxidative stress (Tewari *et al.*, 2010; D'Hooghe *et al.*, 2013) that may lead to the oxidation of proteins, which it is necessary to remove. In any case, to determine the specific functions of each of the proteases analysed under the evaluated conditions, other studies that exceed this work would be necessary.

Particularly for *HvSAG12*, several reports indicate that this gene is specifically activated during the last stages of plant senescence (Gan and Amasino, 1997; Noh and Amasino, 1999; Gregersen *et al.*, 2013; Avice and Etienne, 2014) and in some cases its expression was not detectable in non-senescent tissues (Lohman *et al.*, 1994; Noh and Amasino, 1999). However, in this work we observed not only detectable expression of *HvSAG12* in the green leaves of both well-supplied and S-deficient plants (Figs. 1B, 3B, and 4A) but also an induction due to S deficiency (Fig. 3B). On the other hand, that *HvSUBT* presented a senescence associated induction by S deficiency during monocarpic senescence (Fig. 4C) but not during the sequential leaf senescence (Fig. 1E, 2E) indicates it as an interesting subject for further studies to evaluate its implications at the whole plant level and in the final grain protein content and composition.

In summary, it is concluded that the delay in leaf senescence due to S deficiency is accompanied by a delay in the induction of the gene expression of several proteases that could not be completely overcome with the application of senescence inductors such as MJ + darkness. However, the achieved induction was able to reverse the inhibition of phloem amino acid remobilization caused by S deficiency, which could ultimately alter the stress symptom in younger leaves. In addition, neither S deficiency nor MJ + darkness exerts the same effect on all proteases, suggesting the involvement of different transduction pathways triggering the induction of each one. Besides, the S effect varies depending on leaf age and plant

developmental stage and on the nature of S deficiency, an observation that has not been reported before. These results increase the volume of knowledge on the regulation of the expression of the analysed proteases and exhibit the complexity of the metabolic regulations that are induced during stress conditions, since the effects produced on crop growth and development might depend on the stress intensity and duration.

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LEGEND TO THE FIGURES

Figure 1. Relative gene expression of (A) *HvPAP*, (B) *HvSAG12*, (C) *HvALEU*, (D) *HvLEGU*, (E) *HvSUBT*, (F) *HvCPMIII*, (G) *HvCND41* and (H) *HvLEU-AP* in source leaf of S-starved (white bars) and control (black bars) plants at 11 and 28 days after S treatment (DAT) from experiment 1. Data represent mean ± SE, n = 3. *P*-values of significance between time (T), sulphur treatment (S) and their interaction calculated by a two-way *ANOVA* are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Letters representing significant differences calculated by a LSD post-hoc analysis are shown when there is TxS interaction.

Figure 2. Relative gene expression of (A) *HvPAP*, (B) *HvSAG12*, (C) *HvALEU*, (D) *HvLEGU*, (E) *HvSUBT*, (F) *HvCPMIII*, (G) *HvCND41* and (H) *HvLEU-AP* in source leaf of S-starved (white bars) and control (black bars) plants at 0, 2 and 3 days after methyl-jasmonate + darkness incubation (DAI) from experiment 2. Data represent mean ± SE, n = 3. *P*-values of significance between time (T), sulphur treatment (S) and their interaction calculated by a two-way *ANOVA* between 0 and 2 DAI are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Letters representing significant differences calculated by a LSD post-hoc analysis are shown when there is TxS interaction. Asterisk (*) indicates significant differences ($p \leq 0.05$) between S-starved plants at 2 and 3 DAI calculated by a one-way *ANOVA*.

Figure 3. Relative gene expression of (A) *HvPAP*, (B) *HvSAG12*, (C) *HvALEU*, (D) *HvLEGU*, (E) *HvSUBT*, (F) *HvCPMIII*, (G) *HvCND41* and (H) *HvLEU-AP* in source leaf of plants submitted to low (LS, white bars) and high (HS, black bars) S availability at 16 DAS from experiment 3. Data represent mean ± SE, n = 3. Asterisk (*) indicates significant differences ($p \leq 0.05$) between treatments calculated by a one-way *ANOVA*.

Figure 4. Relative gene expression of (A) *HvSAG12*, (B) *HvLEGU* and (C) *HvSUBT* in source leaves from S-starved (white circles) and control (black squares) plants during grain filling at 55, 69, 78 and 84 days after S treatment (DAT) from experiment 4. Data represent mean ± SE, n = 3. *P*-values of significance between time (T), sulphur treatment (S) and their interaction calculated by a two-way *ANOVA* are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Letters representing significant differences calculated by a LSD post-hoc analysis are shown when there is TxS interaction.

Figure 5. Phloem amino acids exudated from source leaf of S-starved (white bars) and control (black bars) plants at 11 and 28 days after S treatment (DAT) from experiment 1 (A) and at 0, 2 and 3 days after methyl-jasmonate + darkness incubation (DAI) from experiment 2. Data represent mean ± SE, n = 4. *P*-values of significance between time (T), sulphur treatment (S) and their interaction calculated by a two-way *ANOVA* between 11 and 28 DAT (A) and 0 and 2 DAI (B) are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Letters representing significant differences calculated by a LSD post-hoc analysis are shown when there is TxS interaction. Asterisk (*) indicates significant differences ($p \leq 0.05$) between S-starved plants at 2 and 3 DAI calculated by a one-way *ANOVA*.

Table 1. List of the primers used. The name of the gene, the melting temperature (Tm), the gene forward and reverse sequences, the reference of the work from which each gene was obtained and the GenBank accession number are detailed. Primer sequences with the symbol (†) were designed by the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

Gene abreviation	Name	Tm (°C)	Forward (5`- 3`)	Reverse (5`- 3`)	Reference	Accession N°
Hv.EF1-□	Elongation factor 1-alpha	60	AGGTCCACCAACCTTGACTG	CAACAGGCACAGTTCCAATG	Nielsen et al., 1997	Z50789
Hv.Actin	Actin	62	GTATGGAAACATCGTGCTCAGTGG	CTTGATCTTCATGCTGCTCGGA	Hansen et al., 2009	AY145451
Hv.PAP	Papain	58	AAAGGTGGCAAGGATTATTGG	TGCCACAGATACCTGACGAT	Parrott et al., 2007; 2010	AM941122
Hv.SAG12	Senesce-associated gene 12	60	ACGAGGAGCGAGCTATCATT	GACCATTGTACACGCCATTC	Parrott et al., 2007; 2010	AK366134
Hv.ALEU	Aleurain	62	CAACAGCTGGTTGACTGTGC	AAGGGTAGGACTCCTCGGTG	This work†	X05167
Hv.LEGU	Legumain	62	TCATGAAGAAGGGCGGGTTG	TGTTGGTGTTCACGTCCGCC	This work†	AM941115
Hv.SUBT	Subtilisin	60	CAGAAAACGCAGGAGTACGT	ATCGACCCGAACGTGTAC	Parrott et al., 2007;	AK36593

				TT	2010	3
Hv.CPMIII	Carboxi-peptidase III	60	CCAAGCAATGCTCACAGACT	GAGTGGACCCACCTTGAGTT	Parrott et al., 2007; 2010	Y09604
Hv.CND41	Chloroplast nucleoid DNA-binding protein 41	58	TCTTCGGGTTCAAGTCCAA	CCTAGCTAGCCACCCTTCAT	Parrott et al., 2007; 2010	AK354049
Hv.LEU-AP	Leucine-aminopeptidase	60	TACCAGCGTCGCTATTGTTC	TCGACTCAGCCTTGAATCTG	Parrott et al., 2007; 2010	AK373799

Table 2. Leaf and plant phenological stages, aerial biomass as dry weight (DW), chlorophyll content as greenness index (GI), soluble protein and leaf amino acid content in source leaves for each treatment and experiment at sample date (days after sowing, DAS; days after S treatment, DAT; days after incubation with MJ + darkness, DAI). Data represents mean of four biological replicates. *P*-values of significance between time (T), sulphur treatment (S) and their interaction calculated by a two-way *ANOVA* are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Letters representing significant differences calculated by a LSD post-hoc analysis are shown when TxS interaction was significant. AS, advance senescence; D, dead leaf; ES, early senescence; FW, fresh weight; GR, green; HS, high sulphur; LS, low sulphur; MS, middle senescence; R, reproductive; V, vegetative.

Experiment	Plant stage	Sample date			Treatment	Leaf stage	Aerial biomass [g DW]	Greenness index [SPAD]	Soluble protein [mg.g^{-1} FW]	Leaf amino acid [□mol.g^{-1} FW]
		[DAS]	[DAT]	[DAI]						
Experiment 1: *S starvation during vegetative stage*	V	21	11	-	S-starved	GR	0.41^{c}	36.4^{a}	7.72	19.89^{a}
					control	GR	0.40^{c}	37.1^{a}	8.08	20.10^{a}
		38	28	-	S-starved	MS	1.09^{b}	19.6^{b}	2.58	10.32^{b}
					control	AS	1.50^{a}	12.0^{c}	3.36	17.62^{a}
					ANOVA		**T *p* = 0.00 S *p* = 0.02 TxS *p* = 0.02**	**T *p* = 0.00 S *p* = 0.00 TxS *p* = 0.00**	**T *p* = 0.00** S *p* = 0.06 TxS *p* = 0.44	**T *p* = 0.00 S *p* = 0.00 TxS *p* = 0.00**
Experiment 2: *Senescence induction in S-starved plants*	V	26	16	0	S-starved	GR	0.75	28.2	4.71^{b}	11.38^{e}
					control	ES	1.03	23.1	5.12^{a}	17.30^{d}
		-	-	2	S-starved/MJ	MS	-	11.5	2.78^{d}	36.46^{c}
					Control/MJ	AS	-	3.9	3.22^{c}	50.22^{a}
		-	-	3	S-starved/MJ	AS	-	5.4	2.27^{e}	40.23^{b}
					Control/MJ	D	-	-	-	-

					ANOVA		**S *p* = 0.00**	**T *p* = 0.00** **S *p* = 0.00** TxS *p* = 0.33	**T *p* = 0.00** **S *p* = 0.00** **TxS *p* = 0.00**	**T *p* = 0.00** **S *p* = 0.00** **TxS *p* = 0.00**
Experiment 3: *Steady-state S deficiency*	V	16	16	-	LS	GR	0.32^{d}	34.4^{b}	3.53	39.99
					HS	GR	0.53^{cd}	42.9^{a}	7.96	15.81
		28	28	-	LS	MS	0.60^{cd}	11.4^{c}	-	-
					HS	AS	1.67^{b}	7.8^{d}	-	-
		35	35	-	LS	AS	0.70^{c}	8.0^{d}	-	-
					HS	D	2.52^{a}	-	-	-
					ANOVA		**T *p* = 0.00** **S *p* = 0.00** **TxS *p* = 0.00**	**T *p* = 0.00** S *p* = 0.20 **TxS *p* = 0.00**	**S *p* = 0.00**	**S *p* = 0.00**
Experiment 4: *S starvation during reproductive stage*	R	106	55	-	S-starved	GR	37.07	43.9^{ab}	11.68	26.99
					control	GR	37.49	46.1^{a}	14.09	28.65
		120	69	-	S-starved	GR	52.57	40.3^{b}	10.45	29.96
					control	GR	60.84	44.7^{ab}	12.72	26.21
		129	78	-	S-starved	ES	57.21	34.2^{c}	6.25	16.82
					control	ES	69.12	31.1^{c}	8.64	24.56
		135	84	-	S-starved	MS	68.48	26.0^{d}	5.78	16.19
					control	AS	80.60	15.6^{e}	6.98	26.64
					ANOVA		**T *p* = 0.00** **S *p* = 0.03** TxS *p* = 0.66	**T *p* = 0.00** S *p* = 0.13 **TxS *p* = 0.00**	**T *p* = 0.00** **S *p* = 0.00** TxS *p* = 0.68	**T *p* = 0.03** S *p* = 0.07 TxS *p* = 0.10

Table 3. Methyl-jasmonate (MJ) + darkness effect on the gene expression of the proteases of S-starved at 3 DAI versus control at 2 DAI plants. *P*-values calculated by a one-way *ANOVA* are shown. Significant values ($p \leq 0.05$) are highlighted in bold. Up arrow indicates induction, down arrow indicates repression, and equal sign indicates no significant effects of S-starved with respect to control plants. DAI, days after incubation with MJ + darkness.

	Control at 2 DAI vs. S-starved at 3 DAI	
Gene	**effect**	***p*-value**
HvPAP	=	0.58
HvSAG12	=	0.36
HvALEU	↑	**0.05**
HvLEGU	=	0.24
HvSUBT	=	0.16
HvCPMIII	↑	**0.02**
HvCND41	↓	**0.00**
HvLEU-AP	↓	**0.04**

Table 4. Relative gene expression of *HvSAG12*, *HvLEGU* and *HvSUBT* at 28 and 35 days after S treatment (DAT) in low sulphur (LS) and high sulphur (HS) source leaves. Percentage of increment of each gene between 16 and 28 DAT for HS plants and between 16 and 35 DAT for LS plants from experiment 3 is shown. Letters indicate significant differences ($p \leq 0.05$) calculated by a one-way *ANOVA* between LS and HS treatments for each gene and sample time (DAT). Asterisk (*) indicates significant differences ($p \leq 0.05$) calculated by a one-way *ANOVA* between expression level of HS at 28 DAT and LS at 35 DAT for each gene.

		[Relative Expression]		
Gene	**Treatment**	**28 DAT**	**35 DAT**	**Expression increase percentage (%)**
HvSAG12	LS	0.325^{b}	0.470*	58^{b}
	HS	2.763^{a}	-	97^{a}
HvLEGU	LS	1.059^{b}	1.267*	35^{b}
	HS	8.870^{a}	-	96^{a}
HvSUBT	LS	0.014^{b}	0.019*	84^{b}
	HS	0.119^{a}	-	99^{a}

Figure 1

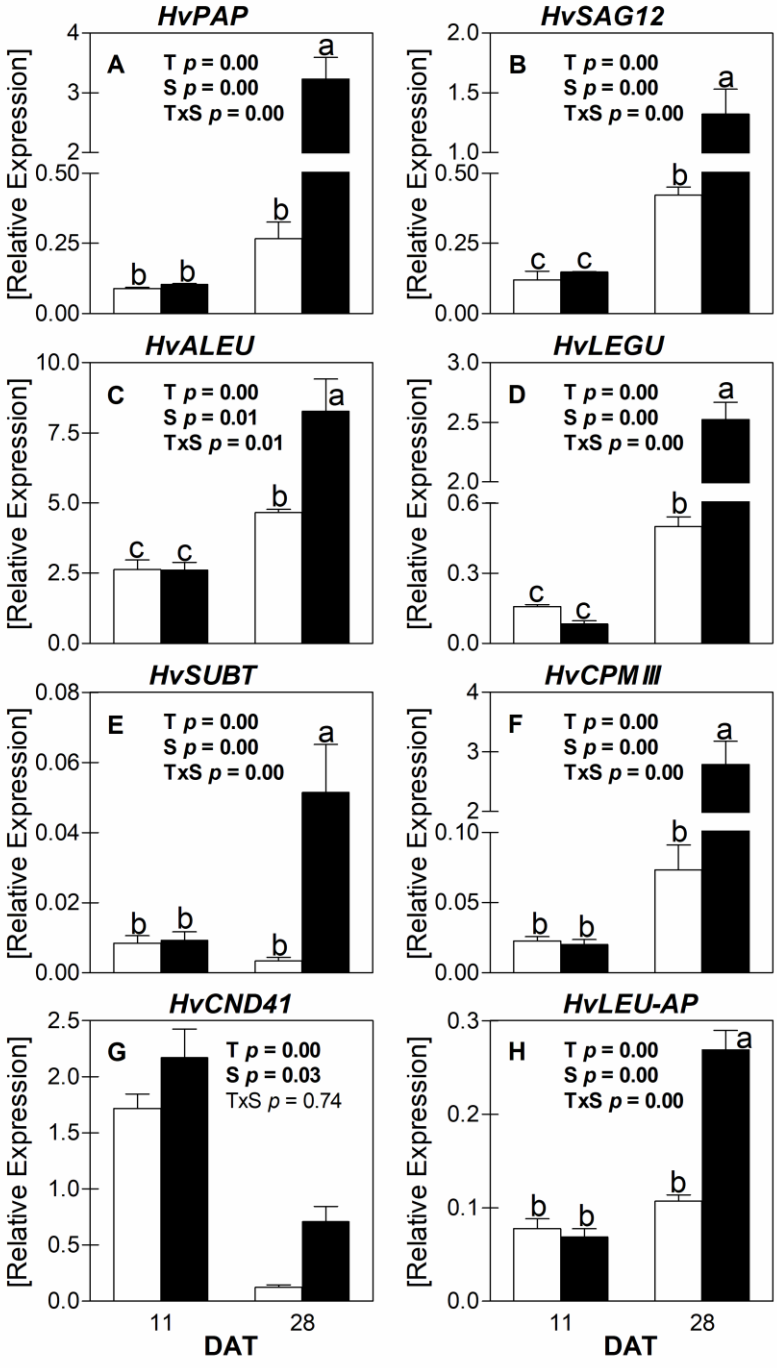

Figure 2

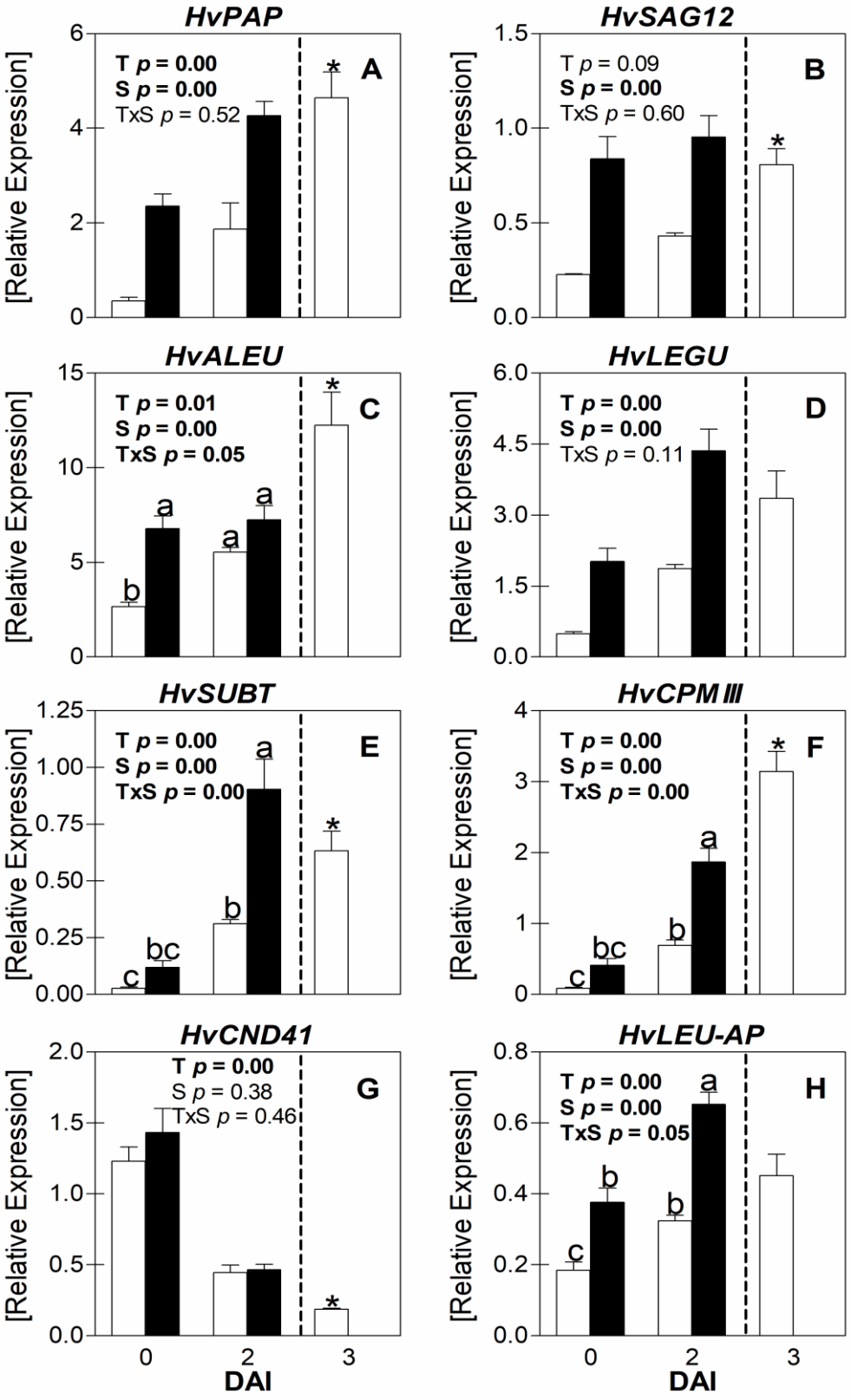

Figure 3

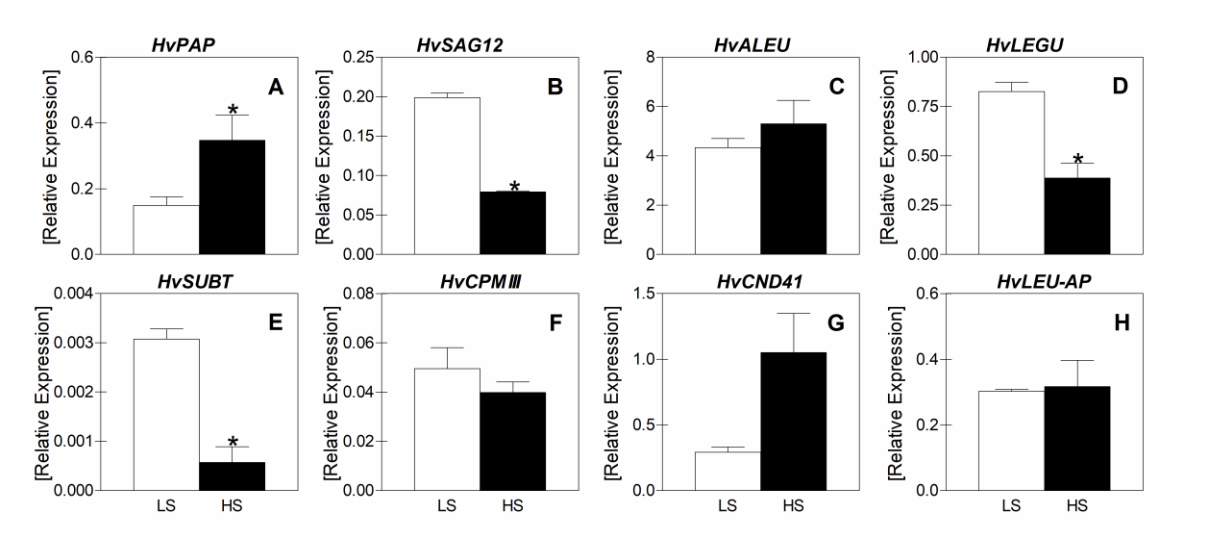

Figure 4

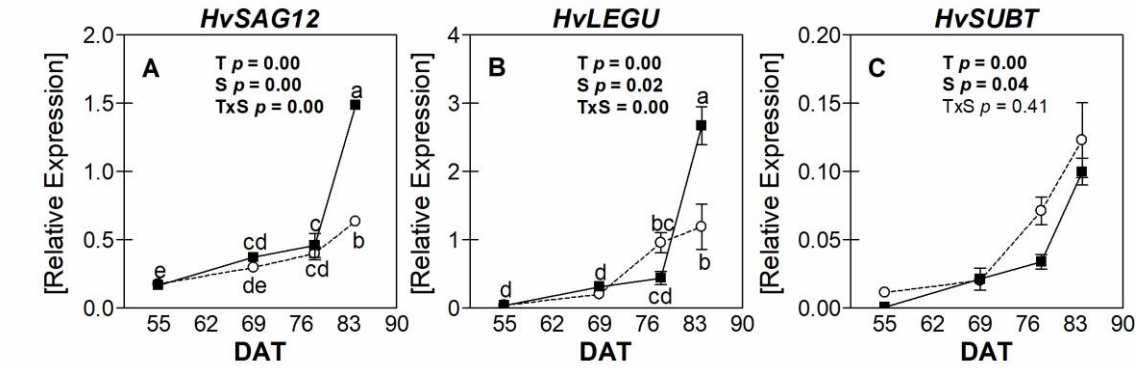

Figure 5