Sulphur deficiency inhibits nitrogen assimilation and recycling in barley plants

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Abstract

Sulphur (S) is incorporated into diverse primary and secondary metabolites that play important roles in proper growth and development of plants. In cereals, a fraction of the nitrogen (N) accumulated in developing grains is guaranteed by amino acid remobilization from vegetative tissues, a contribution that becomes critical when soil nutrients are deficient. Glutamine synthetase (GS) and amino acid transporters (AAT) are key components involved in N assimilation and recycling. The aim of the present study was to evaluate the effect of S availability on the expressions of *HvGS* and several selected *HvAAT* genes in barley plants and on the phloem exudation rate of amino acids. To this end, two independent experiments were designed to impose low S availability conditions to barley plants. Low S availability caused a decrease in the phloem exudation rate of amino acids as well as in the gene expression of all the *HvGS* genes and five of the six *HvAAT* genes analysed. The strong correlation found between the phloem amino acid exudation rate and *HvGS1-1, HvGS1-2, HvAAP7*, and *HvProT1* gene expression may indicate the participation of these genes in the regulation of amino acid remobilization through the phloem.

Additional key words: amino acid transporters, glutamine synthetase, *Hordeum vulgare,* phloem amino acid exudation.

Introduction

Sulphur (S) is a macronutrient required for proper growth and development of plants. Its inorganic form (mainly sulphate, SO_4^{-2}) is assimilated and incorporated into organic compounds such as amino acids (cysteine and methionine) and later into proteins. The disulphide bonds formed in proteins between the thiol groups of cysteine residues play crucial roles in forming and maintaining the tertiary structures and function of proteins. Besides, S is incorporated into diverse primary and secondary metabolites, such as S-adenosylmethionine, S-methyl-methionine, [Iron-S] clusters, hormones, and enzyme cofactors, which play many important roles (see Lewandowska and Sirko 2008).

Over the past decade, S has decreased in several areas due to the reduction of atmospheric deposition as a consequence of a greater control in S emissions and the use of low S fertilizers (Scherer 2001). This reduction has led to insufficient S supply to several crops, generating a reduction in plant growth, vigour, and resistance to abiotic and biotic stresses (see Lewandowska and Sirko 2008). S deficiency not only affects crop yield but also the protein composition of seeds, with a direct impact on the malting quality of barley grains (Zhao *et al.* 2006). Also, it has been reported that S deprivation results in a disruption of nitrogen (N) metabolism, for example by the reduction of nitrate reductase activity (De Bona *et al.* 2011, Sorin *et al.* 2015) or by a limitation of protein synthesis (Hesse *et al.* 2004).

In cereals, a fraction of the N required by developing grains is guaranteed by protein degradation in vegetative organs and amino acid remobilization through the phloem. In particular, when nutrients are deficient in soils, this remobilization process becomes critical to define the final grain protein content (Dalling 1985, Bazargani *et al.* 2012). In a previous work, we reported a lower phloem exudation rate of amino acids in control barley plants grown under field conditions compared to S-fertilized ones (Veliz *et al.* 2014).

Glutamine synthetase (GS) is a key enzyme in N assimilation and recycling due to its role in fixing

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Abbreviations: AAP - amino acid permease; AAT - amino acid transporters; DAS - days after sowing; DOT - days of treatment; GS - glutamine synthetase; HS - high sulphur; LS - low sulphur.

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ammonium to form glutamine. In higher plants, there are two isoforms of this enzyme: a cytosolic GS (GS1, coded by a multigene family) and a plastidic GS (GS2, coded by a single gene). One of the cytosolic GS1 isoforms (GS1-1) is thought to be involved in N remobilization from senescing leaves during grain filling in annual species, mainly due to its localization in vascular tissues (both in the xylem and phloem of leaves and stems), and transfer cells adjacent to the scutellum in developing grains, as reported by Goodall *et al.* (2013). Thus GS1-1 modulation by N availability (Bernard and Habash 2009) is important during senescence (Miflin and Habash 2002) and grain filling (Tabuchi *et al.* 2005, Martin *et al.* 2006). Besides, in young wheat plants, Caputo *et al.* (2009) observed that the expression of *GS1-1* gene correlates with the phloem amino acid export rate. On the other hand, Goodall *et al.* (2013) associated barley GS1-2 with N remobilization during senescence, as well as with N primary assimilation and photorespiration, due to its predominant localization in leaf mesophyll cells, cortex, and pericycle in the roots.

The transport of amino acids through the phloem requires the activity of amino acid transporters (AAT) to ensure their loading into vascular tissues. *AAT* genes are grouped into several gene families, from which the amino acid permease (AAP) family is considered to enclose the main candidates to mediate apoplastic loading of amino acids (Tegeder 2012). Information about the mechanisms that regulate *AAT* gene expression is scarce since to date only a few of them have been fully characterized and tested under abiotic or biotic stresses that could affect their expression (Pratelli and Pilot 2014). Moreover, none of the previous studies concern S availability. In barley, Kohl *et al.* (2012) reported a phylogenetic analysis of 53 *AAT* genes in relation to the known members of other species and determined that only six of them (four being AAP) are highly expressed in flag leaves, a main source of grain N supply.

The aim of the present work was to evaluate the effect of S deficiency on the expression of *GS* genes and those *AAT* genes known to be highly expressed in flag leaves and their relation with the phloem exudation rate of amino acids to deepen the knowledge of the regulation of N remobilization. Progress in this topic is of importance to improve grain quality without increasing fertilizer use.

Materials and methods

Plants and treatments: Barley (*Hordeum vulgare* L. cv. Scarlett) plants were grown in 300-cm^3 pots with *Vermiculite* in a growth chamber (a 16-h photoperiod, an irradiance of 350 μmol m^{-2} s^{-1}, day/night temperatures of 24/18 °C, and a relative humidity of 65 %). Each pot containing five plants represented one independent biological replicate. Plants were watered periodically with nutrient solution containing 1 mM K_2SO_4, 0.2 mM $MgSO_4 \cdot 7 H_2O$, 1 mM $CaCl_2 \cdot 2 H_2O$, 0.2 mM KH_2PO_4, 10 mM KNO_3, 0.08 % (m/v) Na_2EDTA, 0.03 % (m/v) $FeCl_3 \cdot 6 H_2O$, 3 μM H_3BO_3, 0.03 μM $Na_2MoO_4 \cdot 2 H_2O$, 0.75 μM $ZnSO_4 \cdot 7 H_2O$, 0.1 μM $MnSO_4 \cdot 1 H_2O$, 0.2 μM $CuSO_4 \cdot 5 H_2O$ and 0.2 μM $CoSO_4 \cdot 7 H_2O$. Thus, the standard nutrient solution contained S (SO_4^{2-}) in concentration of 1.2 mM and was adopted as the high S (HS; control) in all experiments. To obtain the S-depleted plants and S-starved plants, in low S (LS) solution the K_2SO_4 and $MgSO_4 \cdot 7 H_2O$ were replaced by 1 mM KCl and 0.2 mM $MgCl_2 \cdot 6 H_2O$. Thus, the final concentration of S in this solution was 1.25 μM S (SO_4^{2-}) contributed only by the micronutrient salts. Two independent experiments were performed to impose low S availability conditions. Each experiment was repeated twice in the same conditions, both giving similar results.

Steady-state S deficiency: From the beginning of the experiment, plants were divided into two groups low S (LS) and high S (HS) plants. Along the whole experiment, LS plants were supplied with a low concentration of S (1.25 μM) and HS plants were supplied with the standard concentration of S (1.2 mM) in the nutrient solution. Twenty-two days after sowing (DAS), shoots were collected and dried at 60 °C for 48 h for dry mass (DM) determination. Complementarily, fresh plant material from another group of plants was collected and divided into source tissue (last expanded leaf) and sink tissue (all developing tissues above the source leaf), frozen in liquid N_2, and stored at -80 ºC for further biochemical and molecular analyses. The chlorophyll content (greenness index) of collected leaves was estimated *in vivo* by using a *SPAD* (Soil Plant Analysis Development) analyser (*Konica-Minolta*, Japan).

S starvation: On the beginning of the experiment, plants were supplied with 1.2 mM of S. At 10 DAS (when the 3rd leaf emerged), plants were divided into two groups: control and S-starved. From that moment (days of treatment, DOT = 0), control plants were maintained in the same growing conditions and S-starved plants were supplied with 1.25 μM of S. Samples of the 3rd leaf (source tissue) were collected at 11, 14, 21, and 28 DOT, comprising from the moment of leaf full expansion until complete senescence. These samples were weighed, frozen in liquid N_2 and stored at -80 ºC for further biochemical and molecular analyses. Complementarily, total aerial biomass (DM) and the greenness index of the 3rd leaf were determined from another group of plants at 11, 14, 21, 28 and 32 DOT. At 28 DOT, the greenness index of 4th and 5th leaves was also determined. Besides, samples of developing tissues (sink tissue) were collected, frozen in liquid N_2 and stored at -80 ºC, only

before full expansion of the immediately superior leaf (4th) at 14 DOT (24 DAS).

Phloem exudates were obtained from fully expanded leaves (source tissue), immediately after harvest, 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 25 ºC in the dark to obtain an exudation solution. The exudation solution was stored at -20 °C for further amino acid determination by the ninhydrin method (Yemm and Cocking 1955).

Biochemical analyses: Plant material was ground with liquid N_2 in mortar. Ground tissues (250 mg of fresh mass) were homogenized in 1 cm^3 of 25 mM Tris-HCl buffer (pH 7.5) and 1 % (m/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10 000 *g* and 4 ºC for 30 min. The supernatant was used for colorimetric determinations of soluble proteins (Bradford 1976), free amino acids (Yemm and Cocking 1955) and nitrate (Cataldo *et al.* 1975), using bovine serum albumin, glycine, and KNO_3 as standards, respectively. Measurement of free amino acids and nitrate involved a protein removal step with 5 % trichloroacetic acid and centrifugation at 10 000 *g* for 10 min.

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 by spectrophotometry (*NanoDrop*™, *Thermo Scientific*, USA) and electrophoresis in agarose gels. Total RNA (1 μg) treated with DNAse was used for cDNA synthesis by reverse transcription with *M-MLV* reverse transcriptase (*Promega*, USA), 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 in the *Stratagene Mx3000P 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, 62 °C for 30 s, and 60 °C for 1 min) and 1 cycle of dissociation (95 ºC for 1 min, 55 ºC for 30 s and 95 ºC for 30 s). GS genes (*HvGS1-1, HvGS1-2* and *HvGS2*), AAT genes (*HvAAP2*, *HvAAP4*, *HvAAP6*, *HvAAP7*, *HvLHT2* and *HvProT1*), and the reference genes *H. vulgare* actin mRNA (*HvActin*) and Elongation Factor 1-α (*HvTEF*) were amplified using gene-specific primers manufactured by *Eurofins* (USA). Primer sequences are shown in Table 1 Suppl. The comparative threshold cycle method (ΔΔCt) was applied for relative quantification of gene expression using the *Stratagene Mx3000Pro* thermocycler software®.

Statistical analysis: Analysis of variance (*ANOVA*) and multiple comparison analysis of the data were performed. A Fisher's least significant difference (LSD) test was used for post-hoc comparisons of means. *P*-values were considered significant when they were lower than 0.05. When the time (T) variable was linear, the T × S interaction was also tested by a contrast test. Five independent replicates per treatment were used for biochemical analysis and three for molecular analysis. Besides, for S starvation experiment, a Pearson correlation was carried out to analyse possible correlations of each gene expression with the phloem exudation rate of amino acids (Table 2 Suppl.).

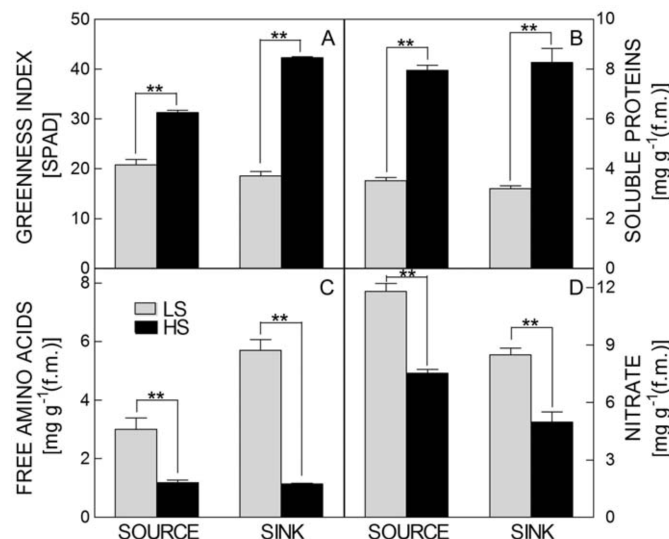

Fig. 1. Greenness index (*A*), soluble proteins (*B*), free amino acids (*C*), and nitrate (*D*) content in source and sink tissues of plants cultivated under low (LS) or high (HS) S availability. Means ± SEs, *n* = 5; ** - significant differences at $P < 0.01$ (one-way *ANOVA*).

Results

When plants were grown under two different S availabilities from the beginning of the experiment, the total dry mass of shoots of LS plants was significantly lower than that of HS plants ($P < 0.01$), showing a clear

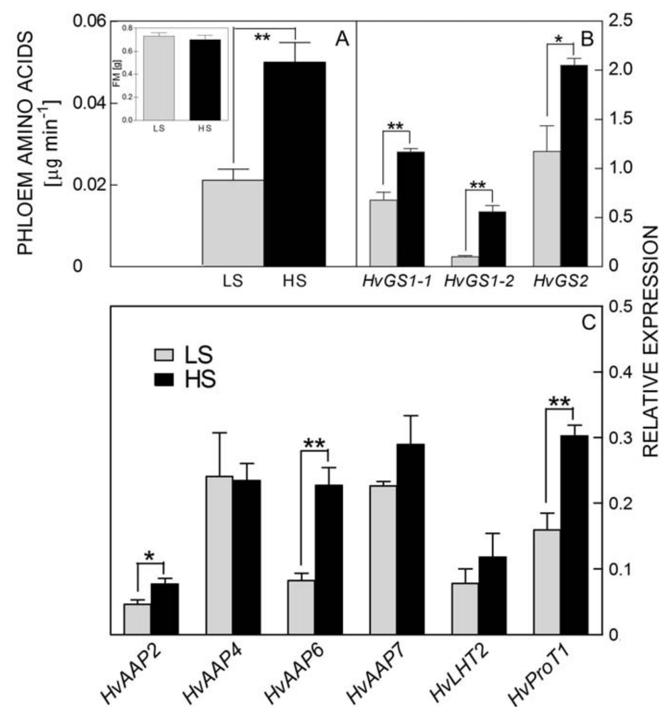

Fig. 2. Phloem amino acid exudated from the 3rd leaf (*A*) and relative expression of *HvGS1-1*, *HvGS1-2*, and *HvGS2* (*B*) and *HvAAP2*, *HvAAP4*, *HvAAP6*, *HvAAP7*, *HvLHT2*, and *HvProT1* (*C*) genes in source tissue of plants cultivated under low (LS) or high (HS) S availability. Means ± SEs, $n = 5$ for phloem amino acids and $n = 3$ for gene expression; *, ** - significant differences at $P < 0.05$ and $P < 0.01$, respectively (one-way *ANOVA*). Inset shows fresh mass (FM) of source leaves.

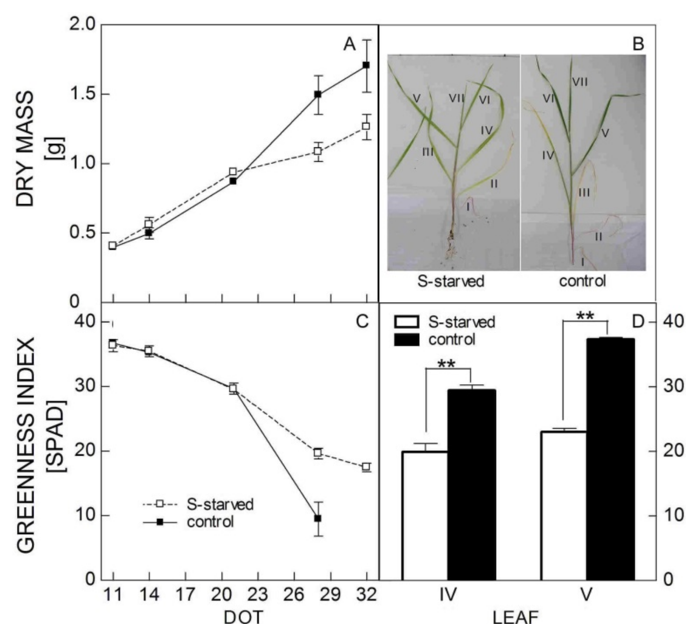

Fig. 3. Total shoot dry mass (DM; *A*) and greenness index of 3rd leaves over time (*C*) of S-starved and control plants. Image of control and S-starved plants at 28 DOT (*B*). Greenness index of 4th and 5th leaves at 28 DOT of S-starved or control plants (*D*). Means ± SEs, $n = 5$;** - significant differences at $P < 0.01$ (one-way *ANOVA*). *P*-values of significance between treatments (S), time (T) and their interaction are: *A* - T $P < 0.01$, S $P < 0.05$, T×S $P < 0.01$ and *C* - T $P < 0.01$, S $P < 0.01$, T×S $P < 0.01$.

reduction in plant growth as a consequence of S deficiency. Chlorophyll (estimated by the greenness index) and protein content of both source and sink tissues were also reduced by S deficiency (Fig. 1*A*,*B*), whereas the content of free amino acids (Fig. 1*C*) and nitrate (Fig. 1*D*) was higher in LS plants than in HS ones. The effect of S deficiency on the greenness index (Fig. 1*A*) and amino acid content (Fig. 1*C*) was higher in sink tissue than in source tissue. Specifically, the greenness index decreased 1.5-fold in source tissue and about 2.3-fold in sink tissue. In the same way, amino acid content increased 2.5-fold and 5-fold in source and sink tissues, respectively. In contrast, the phloem amino acid export rate from source tissue was significantly lower in LS than in HS plants (Fig. 2*A*). Despite the decrease in total shoot dry mass of S-deficient plants, the fresh mass of the source tissue did not vary between treatments (Fig. 2*A*), indicating that the effects observed in the biochemical and molecular parameters were not caused by changes in the leaf mass but they could be explained as a consequence of S availability.

When analysing the amount of mRNA of the different *HvGS* genes in the source tissue, we observed that S deficiency significantly reduced expression of the three *GS* isoforms (*HvGS1-1, HvGS1-2* and *HvGS2*) (Fig. 2*B*): *HvGS1-1* relative expression was reduced about 41 %, *HvGS1-2* expression was reduced about 81 %, and *HvGS2* expression was reduced 42 %. Finally, when analysing the amount of mRNA of the different *HvAAT* genes in source tissue, we observed that *HvAAP2, HvAAP6*, and *HvProT1* relative expression was significantly down-regulated, and that *HvAAP7* showed a tendency to decrease with S deficiency. Meanwhile, no effect of S deficiency was found for *HvAAP4* or *HvLHT2* gene expression (Fig. 2*C*).

Under S starvation, no differences in total shoot DM were observed between control and S-starved plants on day 11 and 14. However, at 21 DOT, S-starved plants showed a significant lower DM than control plants (Fig. 3*A*). At 28 DOT, S-deficient plants showed a pale green colour but their older leaves presented less visible signs of senescence than control plants (Fig. 3*B*). Besides, at 32 DOT, the 3rd leaves of control plants were completely senescent while those of S-starved plants were still alive (Fig. 3*B*,*C*). The greenness index of leaf 3rd leaves decreased gradually in both treatments along time (Fig. 3*C*) until 21 DOT, and thereafter decreased abruptly in control plants while it remained higher in

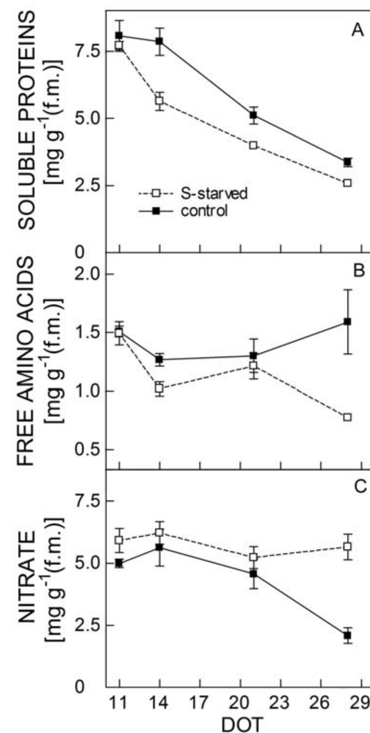

Fig. 4. Soluble proteins (*A*), free amino acids (*B*), and nitrate (*C*) content in source tissue of S-starved and control plants. at different days after treatment (DOT). Means ± SEs, $n = 5$. *P*-values of significance between treatments (S), time (T), and their interaction calculated from a two-way *ANOVA* are: *A* - T $P < 0.01$, S $P < 0.01$, T×S $P < 0.05$; *B* - T $P = 0.11$, S $P < 0.05$, T×S $P < 0.05$, and (C) T $P < 0.01$, S $P < 0.01$, T×S $P < 0.05$.

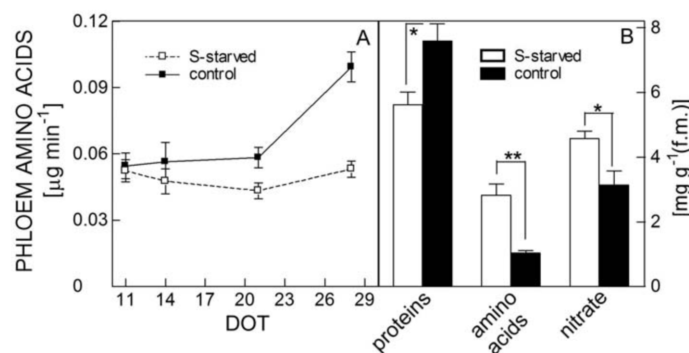

Fig. 5. Phloem amino acid exudated from the 3rd leaf of S-starved and control plants (*A*). Soluble proteins, free amino acids and nitrate content in sink tissue at 14 DOT of S-starved and control plants (*B*). Means ± SEs, $n = 5$;.*,** - significant differences at $P < 0.05$ and 0.01, respectively, obtained from a one-way *ANOVA*. *P*-values of significance between treatments (S), time (T), and their interaction are: A - T $P < 0.01$, S $P < 0.01$, T×S $P < 0.01$.

S-starved plants (Fig. 3*C*). However, the greenness index of the leaves emerged after S depletion (4th and 5th leaves) was lower than of those of control plants (Fig. 3*D*).

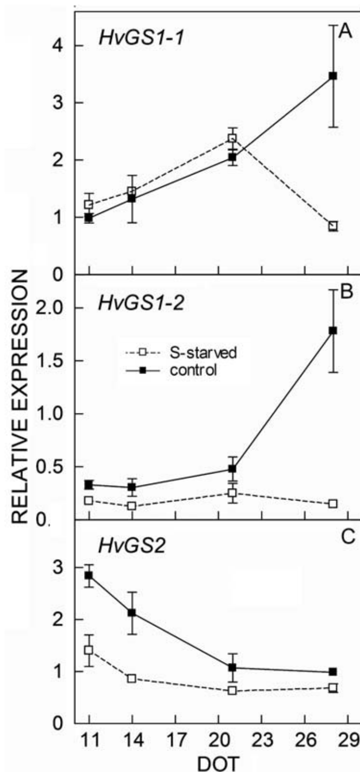

Fig. 6. Expression pattern of *HvGS1-1* (*A*), *HvGS1-2* (*B*), and *HvGS2* (*C*) genes in 3rd leaf of S-starved and control plants. Means ± SEs, $n = 3$; *P*-values of significance between treatments (S), time (T), and their interaction calculated from a two-way *ANOVA* are: *A* - T $P < 0.05$, S $P = 0.08$, T×S $P < 0.01$; *B* - T $P < 0.01$, S $P < 0.01$, T×S $P < 0.01$, and *C* - T $P < 0.01$, S $P < 0.01$, T×S $P = 0.07$.

The content of soluble proteins in the source tissue decreased along time in both treatments, but in S-starved plants this decrease started earlier, showing significantly lower protein content along the whole experiment (Fig. 4*A*). On the other hand, the content of amino acids in the source tissue of control plants remained invariable during the whole experiment whereas it decreased in S-starved plants, which showed a lower amino acid content at 14 and 28 DOT than control plants (Fig. 4*B*). Conversely, nitrate content remained higher and constant in S-starved plants and decreased in control plants (Fig. 4*C*). The phloem exudation rate of amino acids in control plants remained constant until 21 DOT and increased abruptly on the last sampling date (Fig. 5*A*), when the 3rd leaf showed an advanced senescence state (Fig. 3*B*). Lower exudation rates were observed in S-starved plants at all times and no exudation peak was recorded on the last sampling date (Fig. 5*A*). In sink tissue, protein content was lower whereas amino acid and nitrate content was higher in S-starved plants than in control ones at 14 DOT (Fig. 5*B*).

In control plants, *HvGS1-1* expression increased over time (Fig. 6*A*), whereas *HvGS1-2* expression increased abruptly at 28 DOT (Fig. 6*B*), and *HvGS2* expression decreased over time (Fig. 6*C*). S starvation altered the gene expression of all GS isoforms, with an abrupt decrease in *HvGS1-1* expression on the last sampling date (Fig. 6*A*) and lower expressions of both *HvGS1-2* and *HvGS2* compared to control plants (Fig. 6*B*,*C*). When analysing *HvAAT* expression, we found different expression patterns for each gene along time (Fig. 7). In control plants, *HvAAP2* expression decreased over time (Fig. 7*A*), while *HvAAP4* expression initially showed an increase before it decreased (Fig. 7*B*). On the other hand, *HvAAP7* (Fig. 7*D*) and *HvProT1* (Fig. 7*F*) expressions increased with time, while *HvAAP6* (Fig. 7*C*) and *HvLHT2* (Fig. 7*E*) expressions remained constant during the whole experiment. S starvation reduced the expression of *HvAAP4* (Fig. 7*B*), *HvAAP7* (Fig. 7*D*) and *HvProT1* (Fig. 7*F*) in respect to control plants. Besides, *HvAAP6* expression remained constant in S-starved plants until 21 DOT and then decreased significantly (Fig. 7*C*), while the increase in *HvProT1* expression observed in control plants was absent in S-starved plants (Fig. 7*F*). Finally, no significant effect of S starvation was found in *HvAAP2* (Fig. 7*A*) and *HvLHT2* (Fig. 7*E*) expressions.

Finally, to better understand the possible relation of the different *HvGS* and *HvAAT* genes analysed with the phloem exudation rate of amino acids, we performed a Pearson correlation analysis over the results obtained in this experiment, and found a strong correlation between the phloem amino acid export rate and *HvGS1-1*, *HvGS1-2*, *HvAAP7* and *HvProT1* gene expression (Table 2 Suppl.).

Discussion

The decrease in plant DM, chlorophyll, and protein content and the accumulation of nitrate and amino acids observed in this work in S-deficient tissues (Fig. 1; Fig. 3*A*,*D,* 4*A*,*C*, 5*B*) is coincident with previous reports about the effect of S deficiency on plants (Hesse *et al.* 2004, Nikiforova *et al.* 2004, Lunde *et al.* 2008). However, despite the knowledge that chlorophyll, Rubisco, and protein content decrease gradually once senescence has begun (Buchanan-Wollaston *et al.* 2003, Avila-Ospina *et al.* 2015), here we observed a fairly constant exudation rate of amino acids along time, with a strong peak only at the end of the life of control leaves (Fig. 5*A*). This suggests that the N exportation rate does not gradually increase with time, although leaves have

increasing amounts of free amino acids available for remobilization. The lower exudation rate of amino acids observed under S deficiency (Fig. 2*A*, 5*A*) is in agreement with previous results obtained in field conditions, where the rate of amino acids reaching the ears through the phloem was lower in non-S-fertilized than in S-fertilized barley plants (Veliz *et al.* 2014). Moreover, the absence of the exudation peak recorded in control plants (Fig. 5*A*) and the longer life time of S-starved leaves (Fig. 3*B*,*C*) suggest a delay in the senescence progress triggered by S-deficiency as it has been reported in oilseed rape (Dubousset *et al.* 2009). However, further investigations are needed to better understand the regulation of the senescence process by S deficiency in barley plants.

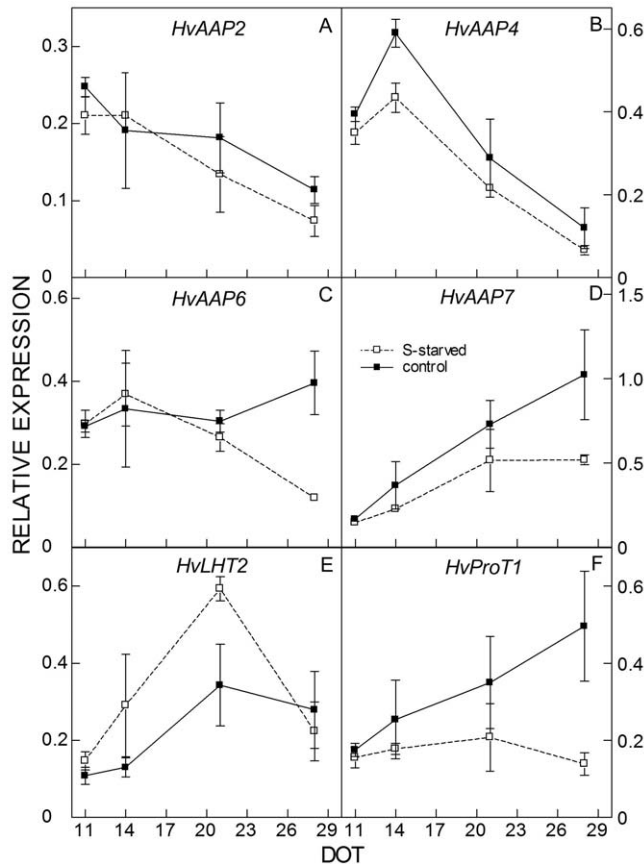

Fig. 7. Expression pattern of *HvAAP2* (*A*), *HvAAP4* (*B*), *HvAAP6* (*C*), *HvAAP7* (*D*), *HvLHT2* (*E*), and *HvProT1* (*F*) genes in 3rd leaf of S-starved and control plants. Means ± SEs, $n = 3$; *P*-values of significance between treatments (S), time (T), and their interaction calculated from a two-way *ANOVA* are: *A* - T $P < 0.01$, S $P = 0.25$, T×S $P = 0.75$; *B* - T $P < 0.01$, S $P < 0.05$, T×S $P = 0.52$; *C* - T $P = 0.47$, S $P = 0.11$, T×S $P < 0.05$; *D* - T $P < 0.01$, S $P < 0.05$, T×S $P = 0.08$; *E* - T $P < 0.01$, S $P = 0.08$, T×S $P = 0.23$, and *F* - T $P = 0.24$, S $P < 0.05$, T×S $P < 0.05$.

At the same time, the expression dynamics of the different GS isoforms during the senescence of control plants (Fig. 6) is supported by previous results showing that the abundance of cytosolic GS (GS1) mRNA increases whereas that of plastidic GS (GS2) decreases in senescing leaves (Bernard and Habash 2009). On the other hand, the three differential expression patterns observed in this work along senescence for the different *HvAAT* genes analysed (Fig. 7) add information about their possible roles during the life-time of the leaf, and point *HvAAP7* and *HvProT1* as potentially implicated in amino acid remobilization during senescence, since their expression increased as senescence progressed (Fig. 7*D,F*). Besides, we demonstrated that a reduction in S availability leads to a significantly lower expression of all the *HvGS* (Fig. 2*B*; Fig. 6) and five of the six *HvAAT* (Fig. 2*C*; Fig. 7) genes analysed. Moreover, the downregulation of *HvGS* genes, the lower content of free amino acids, and the higher content of nitrate in the source tissue of S-starved plants (Fig. 4*B*,*C*) indicate an impairment of N assimilation under the S starvation in an attempt to prevent the non-S-amino acid accumulation in source tissue (Wallsgrove *et al.* 1987, Cren and Hirel 1999, Miflin and Habash 2002, Goodall *et al*. 2013, Yamaya and Kusano 2014). However, it is also possible that the accumulation of free amino acids and nitrate in the sink tissue of S-deficient plants (Fig. 1*C*,*D;* Fig. 5*B*) works as a negative signal for *HvGS* gene expression as well as for the amino acid export, since source and sink metabolism are closely coordinated to maintain the

balance between growth and nutrient supply. This hypothesis is also reinforced by several works demonstrating that S deprivation significantly reduces nitrate uptake, nitrate reductase activity, and root nitrate transporter activity, as a consequence of a negative feedback on nitrate uptake due to the accumulation of non-S amino acids (Abdallah *et al*. 2011, De Bona *et al.* 2011, Sorin *et al.* 2015). Finally, the decrease in *HvGS2* gene expression mediated by S starvation might also be associated with changes in photorespiration rates because this isoform has been shown to be transcriptionally regulated by this metabolic pathway (Edwards and Coruzzi 1989) and there is information that this process is altered by S availability (Chen and Wang 2006, Nikiforova *et al.* 2005).

As expected, the results obtained by the Pearson analysis showed a strong correlation between phloem amino acids and both cytosolic *HvGS1* genes (Table 2 Suppl.), which not only reinforces the hypothesis of a crucial role of GS1 in N remobilization (Caputo *et al.* 2009, Goodall *et al.* 2013, Avila-Ospina *et al.* 2015), but also shows the importance of S nutrition on N remobilization. In addition, the increase in *HvAAP7* gene expression along time observed in control plants (Fig. 7*D*) and the high correlation between phloem amino acid transport and the expression of this *AAT* gene (Table 2 Suppl.) provide new evidence for a significant role of this particular transporter in N remobilization, although no significant sequence homology with known *AAT* genes has been reported for this gene (Kohl *et al.* 2012). On the other hand, the correlation found between *HvProT1* and phloem amino acids (Table 2 Suppl.) was unexpected, since this transporter is assumed to be specific for proline, an amino acid normally present at very low amounts in the phloem sap of several plant species, including barley (Weibull *et al.* 1990, Winter *et al.* 1992, Caputo *et al.* 2001, 2009). However, *HvProT1* expression also correlated with the expression of *HvGS1* genes (*HvProT1* and *HvGS1-1* and *HvProT1* and *HvGS1-2*), in agreement with previous reports of Larher *et al.* (1998) and Brugière *et al.* (1999), who suggested a role of *HvGS1* in the regulation of *HvProT1* expression. These authors observed a decrease in the amount of proline in plant organs and in xylem and phloem saps of *Nicotiana tabacum* transgenic plants lacking GS1, as well as by the application of a GS inhibitor to oilseed rape leaf discs, which prevented the conversion of amino acids to proline. Still, further investigations are needed to elucidate the biological function of *HvProT1* and clarify its possible participation in the regulation of amino acid transport *via* the phloem.

In summary, the inhibition of amino acid remobilization through the phloem in S-deficient plants might be related to a lower expression of *HvGS1-1* and *HvGS1-2* but also to a downregulation of *HvAAP7* and *HvProT1*. However, although *HvAAP6* did not correlate with phloem amino acids, its participation in N remobilization cannot be fully discarded since its gene sequence is closely related to that of *Arabidopsis AtAAP2* and *AtAAP5*, both thought to be involved in the loading of amino acids (especially glutamine) into the phloem (Tegeder and Rentsch 2010). In fact, the constant expression of *HvAAP6* over time in control plants and its significant decrease at 28 DOT triggered by S starvation suggest both an important role of S in the regulation of this gene and its putative involvement in the long-distance transfer of amino acids.

Conclusion

A better understanding of the effect of S availability on different factors regulating the N remobilization becomes necessary to improve grain quality without increasing fertilizer use, as S deficiency is known to affect not only barley yield but also grain malting quality. In this work, we demonstrated that low S availability decreases the phloem exudation rate of amino acids and the gene expression of all *GS* isoforms as well as of five of the six *AAT* genes analysed, two major components in the regulation of amino acid remobilization. This highlights the relevance of an adequate S nutrition to ensure a satisfactory N redistribution throughout the life cycle of the plant. Our results also provide new evidence that not only *HvGS1-1* and *HvGS1-2* but also *HvAAP7* and *HvProT1* may participate in the regulation of amino acid remobilization *via* the phloem. These results reinforce the knowledge of the impact of S deficiency on the disruption of normal N metabolism but also set the focus on two possible N remobilization pathways regulated by S nutrition: a negative feedback due to the accumulation of N metabolites in sink tissue and/or a delay in senescence.

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