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Phloem sugars and amino acids as potential regulators of hordein expression in field grown malting barley (*Hordeum vulgare* L.)

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ABSTRACT

The commercial quality of malting barley is dependent on the content and composition of grain proteins which are subjected to nitrogen (N) and sulfur (S) control. In nutrient deficient soils, grain protein content is mainly a consequence of the remobilization efficiency. In order to evaluate the effect of N and S soil availability on phloem amino acid and sugar export rates and B- and C-hordein gene expression during grain filling, a factorial combination of N and S fertilization assay was carried out under field conditions. Besides, several carbon (C) and N metabolites were analyzed in leaves and spikes. We observed that, even under soil N and S availabilities that do not limit yield, N and S fertilization induced significant changes in N and C metabolism. N phloem remobilization was promoted by S fertilization independently of N fertilization and C remobilization was also promoted by S fertilization but only in N fertilized plants. The B- and C-hordein gene expression correlated positively with sugar and amino acid exudation rate, respectively. Our results suggest an important role of the export rate of sugars and amino acids in the regulation of grain prolamine expression.

1. Introduction

In many agricultural regions of the world, the historical lack of replenishment of nutrients exported by crops and soil erosion has led to a continuous decrease of soil nutrient availability. Nitrogen (N) is the most limiting nutrient for the production of grain crops, due to its high requirement by plants and susceptibility to losses. On the other hand, sulfur (S) fertilization has increasingly become an important issue in crop management; especially after the reduction of atmospheric deposition due to control in S emissions by industry and the use of low S grade fertilizers (Scherer, 2001).

Barley (*Hordeum vulgare* L.) is a widely cultivated cereal crop and is the major raw material for brewing beer. Consequently, grain quality, which is governed by a number of grain properties such as the content and composition of proteins and carbohydrates, is crucial to its commercial use. In deficient soils, N fertilization increases crop yields and plays a dominant role in determining the amount of protein stored in the grain (Prystupa et al., 2003) which for malting purposes should be between 10 and 12% of the grain weight. However, in the new developed barley varieties showing higher yields than the old ones, even the minimum protein content demanded by malting industry is not always achieved.

The prolamins of barley, major grain storage proteins of cereals, are called hordeins and are classified into B, C, D and γ fraction. The B and C fractions account for 70–80% and 10–20%, respectively, of total hordein content, while the D and γ groups are quantitatively minor components. Regarding its composition, the B and γ fractions are S-rich, the D fractions have an intermediate S content and the C fractions are S-poor (See Shewry and Tatham, 1990).

The primary control of prolamin gene expression in wheat and barley is at the level of gene transcription (Giese and Hopp, 1984; Shewry et al., 2001), and it is subject to nutritional control, depending primarily on the availability of reduced N in the spikes. In barley liquid culture spikes, it was observed that an alteration in N concentration produces changes in the level of C-hordein mRNA over short periods, decaying almost 70% under suboptimal N regime (Giese and Hopp, 1984; Qi et al., 2006). S deficiency has also been shown to affect the composition of proteins in barley grains,

Abbreviations: ANOVA, analysis of variance; C, carbon; DAA, days after anthesis; EDTA, ethylene-diamine-tetraacetic acid; FW, fresh weight; N, nitrogen; P, phosphorus; S, sulfur; *T*, time.

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with depletion of S-rich B-hordeins and increment of S-poor C-hordeins (Shewry et al., 1983).

N and S are in part guaranteed by protein degradation in leaf blades and stems, which are remobilized to the grain through the phloem, principally as amino acids (Cliquet et al., 1990). In nutrient deficient soils (such as N or S), grain protein content is to a large extent a consequence of remobilization efficiency (Dalling, 1985; Fitzgerald et al., 2001). However, there is limited information available about the effect of N and S fertilization on the flux assimilated through the phloem and its relation to hordein gene expression in field grown plants.

The aim of this work was to analyze the combined effect of N and S fertilization on the remobilization of C and N and its consequent effect on the expression pattern of B and C-hordeins in field grown plants of barley.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted within a commercial barley (*H. vulgare* L. cv. Scarlett) crop located in the Pampean region (Argentina, 34°38'S, 60°56'W). As there is not a precise soil-based diagnostic method for S availability, the site was chosen considering the characteristics of soil and history of use which is known to increase the chance of S deficiency (Gutiérrez Boem, 2006). In this area, S deficiencies have already been observed in soybean crops (Salvagiotti et al., 2012).

Four treatments resulting from the factorial combination of two rates of S (not fertilized and fertilized with 10 kg ha^{-1} of S as $CaSO_4$), and two of N (not fertilized and fertilized with 60 kg ha^{-1} of N as urea) fertilization at sowing was carried out. Treatments were organized in four complete randomized blocks, and defined as: N0S0 (control plants, without N or S fertilization), N0S1 (fertilized with S), N1S0 (fertilized with N) and N1S1 (fertilized with both, N and S) plants. All plots (20 m^2 each) were fertilized with 30 kg ha^{-1} of P to avoid phosphorus deficiency. One linear meter of shoots per plot were sampled five times from anthesis to physiological maturity (at 4, 17, 25, 34 and 46 days after anthesis, DAA) and dried at 60 °C for 48 h for biomass determination. At maturity, grains were dried at 60 °C for 48 h for yield, N and S content determination. The last two expanded leaves and spikes of 8 plants were sampled at 17, 25 and 34 DAA and frozen in liquid nitrogen and stored at −80 °C for further analysis. The experiment was repeated during two consecutive years (2010 and 2011) with similar results; therefore, only one is presented here.

2.2. Collection of phloem exudates

Phloem exudates were obtained from eight spikes of each treatment with the EDTA (20 mM, pH 8) facilitated technique in a three hour incubation period as described in Caputo and Barneix (1997). Afterward, the exudation solution (5 ml) was stored at −80 °C for further analysis.

2.3. Biochemical analysis

Leaves and spikes (250 mg fresh weight, FW) were ground with liquid N_2 in a mortar. Fresh tissues obtained were homogenized in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant obtained was used for determination of soluble proteins (Bradford, 1976), free amino acids by the ninhydrin method (Yemm and Cocking, 1955) and soluble sugars by the anthrone method (Yemm and Willis, 1954). In phloem exudates, soluble sugars and free amino acids were also determined by anthrone and ninhydrin methods. Grain N content was analyzed by colorimetry of Kjeldahl digest (Baethgen and Alley, 1989) and S content by turbidimetry after digestion with nitric and perchloric acids (San Martín et al., 1987).

2.4. Total RNA extraction, cDNA synthesis and quantitative PCR

Total RNA was extracted from 100 mg of frozen spikes with TRIzol® Reagent (Ambion, by Life Technologies) following the manufacturer's protocol with the following modification: vegetal tissue was incubated for 5 min after TRIzol® Reagent addition, centrifuged for 1 min at 10,000 × g at 4 °C and the supernatant was transferred to a clean tube to continue the extraction protocol. One microgram of DNAse treated total RNA was used for cDNA synthesis by reverse transcription with Promega retrotranscriptase following the manufacturer's protocol. cDNA samples were used as templates to quantify the expression levels of B- and C-hordein genes. Quantitative PCR analysis was carried out with a FastStart Universal SYBR Green Master (ROX) from Roche. 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). Each gene of interest and the reference gene *H. vulgare* actin mRNA, complete cds (Accession number: AY145451) were amplified using gene-specific primers (Hansen et al., 2009) and the comparative Ct (threshold cycles) method (ΔΔCt) was applied for relative quantification of gene expression using the Stratagene Mx3000Pro thermocycler software®.

2.5. Statistical analysis

Analysis of variance and multiple comparison analysis of the data were performed. The *P*-values showing the level of significance of N, S, time (*T*) and their interactions calculated from a three-way ANOVA of Figs. 2–4 are shown in Table 1.

3. Results and discussion

3.1. Plant growth

N fertilization increased shoot biomass, but did not result in any increment in grain yield. S fertilization, instead, did not produce any change in either shoot biomass or grain yield (Fig. 1A). Grain N content, instead, increased with N fertilization (Fig. 1B) and grain S content increased with S fertilization (Fig. 1C). Consequently, soil N and S availability were sufficient to not limit yield but the increment in shoot biomass in response to N fertilization indicates some grade of deficiency.

Table 1
P-values showing the level of significance of N, S, time (*T*) and their interactions calculated from a three-way ANOVA.

	Fig. 2			Fig. 3		Fig. 4			
	A	B	C	A	B	A[a]	B[a]	C	D
N	0.88	0.61	**0.02**	**0.03**	0.12	0.18	**0.02**	0.65	0.47
S	0.84	**0.02**	0.73	**<0.01**	**<0.01**	0.19	0.84	**0.01**	0.41
Time	**<0.01**	**<0.01**	0.85	0.08	**<0.01**	0.07	**<0.01**	**<0.01**	**<0.01**
N × S	**<0.01**	0.64	0.68	0.84	**<0.01**	**0.02**	0.76	0.49	0.85
N × *T*	0.86	0.47	**0.03**	**<0.01**	0.36	0.73	**0.01**	0.48	0.83
S × *T*	**0.05**	0.63	0.41	0.47	0.52	0.57	0.10	**0.05**	0.19
N × S × *T*	0.16	0.52	0.18	0.22	0.08	0.16	0.55	0.75	0.89

$p <$ or $=$ to 0.05 are shown in bold.
[a] Values of the first sampling date were not considered for the ANOVA due to its heterogeneity respect to the others sampling dates.

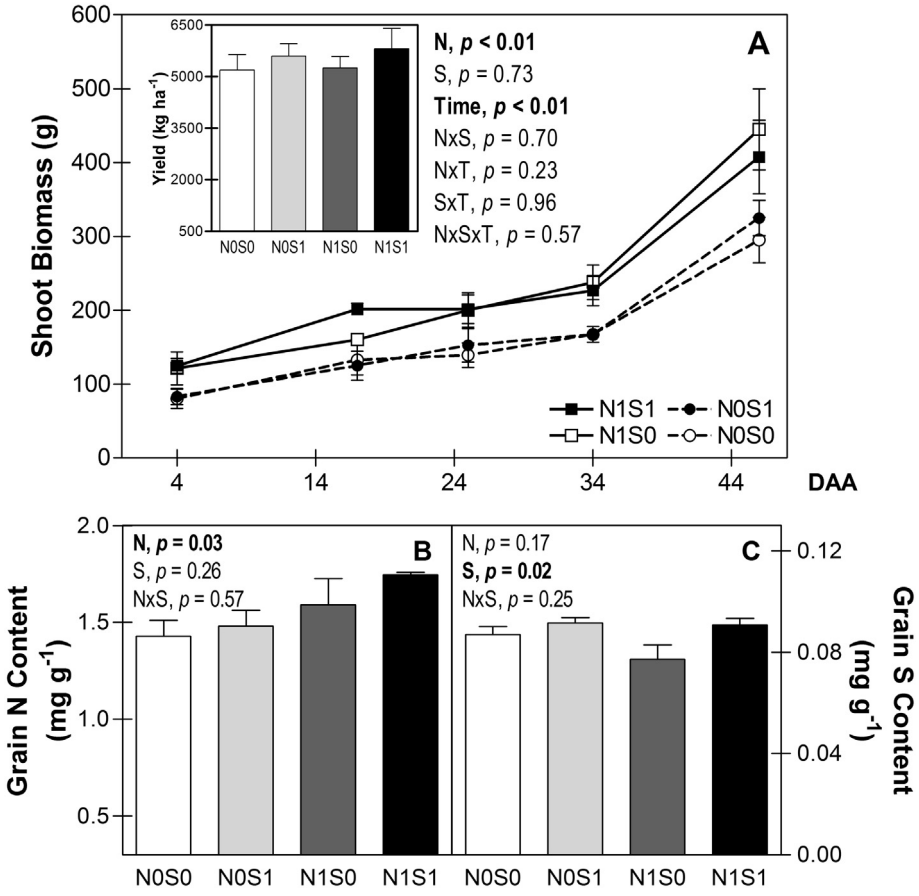

Fig. 1. (A) Biomass of one linear meter of shoots of N1S1 (—■—), N1S0 (—□—), N0S1 (- -●- -) and N0S0 (- -○- -) plants during grain filling; *P*-values show the level of significance of N, S and time (*T*) and their interactions calculated from a three-way ANOVA. Insert graphic represents grain yield at harvest (all interactions were not significant, $P > 0.05$). (B) Grain N content and (C) grain S content of N1S1, N1S0, N0S1 and N0S0 plants at harvest; *P*-values show the level of significance of N, S and N × S interaction calculated from a two-way ANOVA. Data are the means ± SE ($n = 4$). DAA = days after anthesis.

3.2. Leaves biochemical determination

A significant interaction between N × S and a tendency of interaction between S × T were observed for protein concentration (Fig. 2A), thus indicating a different protein degradation dynamics for each treatment. In N fertilized plants, protein concentration in leaves remained constant between 17 and 25 DAA, being higher in S fertilized plants (N1S1), and then both decayed to the same level. In N non-fertilized plants instead, protein concentration decreased constantly in S fertilized plants (N0S1), indicating an acceleration of the senescence process due to N deficiency as was observed in wheat (Criado et al., 2007). However, in not fertilized plants (N0S0), protein concentration increased between 17 and 25 DAA before decay (Fig. 2A), suggesting an S requirement for the occurrence of protein degradation triggered by N deficiency. Based on this result and knowing that thiol-proteases have been related to plant senescence (see Roberts et al., 2012), it would be interesting to evaluate the effect of S fertilization on thiol-protease expression and activity to better understand the effect observed here. In addition, N × S two-way ANOVAs revealed an increment in protein concentration due to S fertilization only at 17 DAA (Fig. 2A), suggesting that S fertilization allowed plants to store more N and S as proteins before the onset of senescence.

At the same time, amino acid concentration in leaves decreased after 25 DAA in N1S1, N1S0, N0S0 treatments, whereas it decreased from the first sampling date in N0S1 plants (Fig. 2B), suggesting again an acceleration of senescence due to N deficiency only in S fertilized plants. At the same time, S fertilized plants showed lower amino acid concentration compared to not fertilized ones (Fig. 2B). It is well documented in the bibliography that under S-deficient conditions, a reduction in protein synthesis is accompanied by an accumulation of non-sulfur amino acids (see Hesse et al., 2004).

The concentration of sugars in leaves remained constant over time for all treatments, but at the first sampling date, N fertilized plants accumulated fewer sugars than non fertilized ones (Fig. 2C).

Nitrate concentration in leaves decreased over time, but it was not affected either by N or by S fertilization (data not shown).

3.3. Phloem exudation determination

Phloem exudation rate of amino acids was higher in S fertilized plants compared to non fertilized ones (Fig. 3A). This effect was observed during the whole experiment in N1S1 plants and only in the first sampling date in N0S1 plants (Fig. 3A). These results along with the fact that S fertilized plants presented lower amino acid concentration in leaves than non fertilized ones (Fig. 2B) suggest that the remobilization of amino acids seems to be promoted by S fertilization independently of the amount of amino acids in leaves. In turn, N fertilization allowed a constant phloem exudation rate of amino acids. On the contrary, N non-fertilized plants presented a higher exudation rate of amino acids than fertilized ones at the beginning of the grain filling period. Then this parameter decreased over time from 17 DAA in S fertilized plants (N0S1) and from 25 DAA in non fertilized plants (N0S0), reaching both a similar

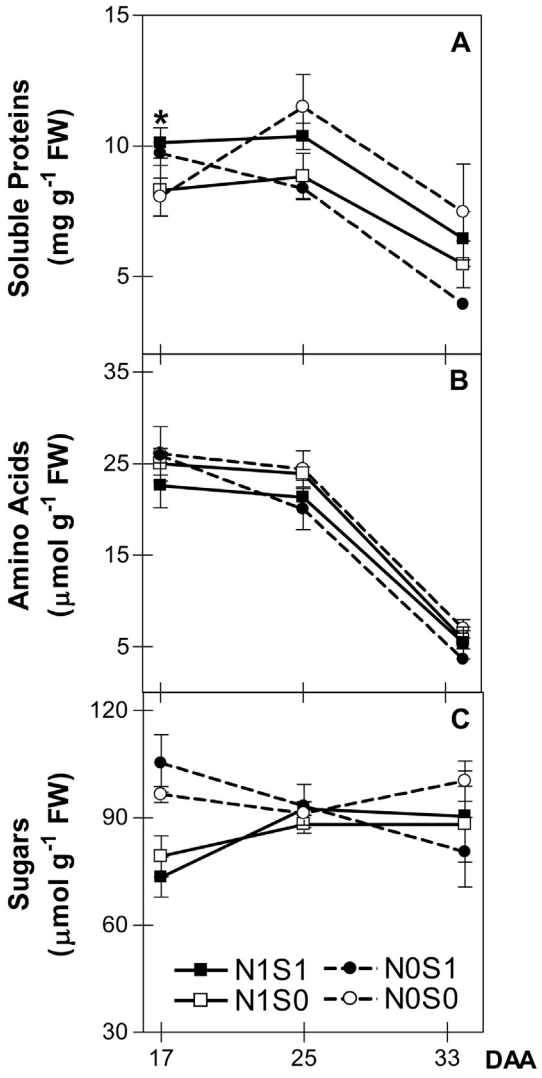

Fig. 2. (A) Soluble protein, (B) amino acid and (C) sugar concentrations in leaves of N1S1 (—■—), N1S0 (—□—), N0S1 (- -●- -) and N0S0 (- -○- -) plants during grain filling. Data are the means ± SE ($n = 4$). The asterisk (*) shows significant differences at 17 days after anthesis (DAA) of S with $P = 0.02$. *P*-values from the three-way ANOVA are shown in Table 1.

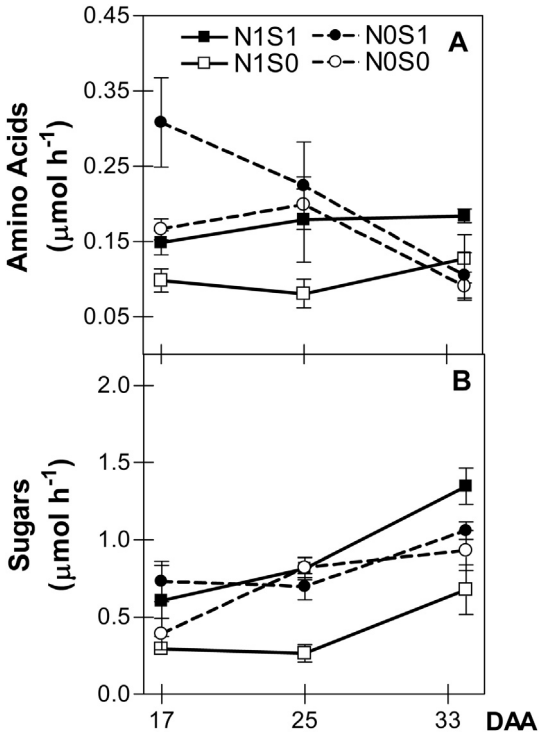

Fig. 3. Phloem exudation rate of (A) amino acids and (B) sugars, obtained from 8 spikes of each treatment, N1S1 (—■—), N1S0 (—□—), N0S1 (- -●- -) and N0S0 (- -○- -), during grain filling. Data are the means ± SE ($n = 4$). DAA = days after anthesis. *P*-values from the three-way ANOVA are shown in Table 1.

value which was lower than that of double fertilized plants (N1S1) (Fig. 3A). In agreement with previous reports (Dalling, 1985; Ercoli et al., 2008), these observations suggest that N remobilization is induced under soil N deficiency. However, it also seems that even moderate N-deficient plants cannot maintain a high exudation rate through all the grain filling period.

The phloem exudation rate of soluble sugars increased over time in all treatments (Fig. 3B). Besides, a significant interaction between both nutrients was found. In N fertilized plants, S fertilization increased sugar exudation rate during all the assay, and at the last sampling date, those plants (N1S1) showed the highest exudation rate of all treatments. However, when N non-fertilized plants were fertilized with S (N0S1 plants), sugar exudation rate was increased only in the first sampling date (Fig. 3B).

Therefore, these results showed that both amino acid and sugar export rate fluctuated in response to N and S availability, but the effects were different for each nutrient. Thus, the export rate of sugars and amino acids seems to be independent of one another, as it was previously reported in wheat (Caputo and Barneix, 1999).

3.4. Spikes molecular and biochemical determinations

The expression profile of the most abundant prolamins in barley grains, B-hordein genes showed an elevated transcription level at 25 and 34 DAA (Fig. 4A), whereas C-hordeins reached their higher expression level at 34 DAA (Fig. 4B), in agreement with the results reported by Hansen et al. (2009). N and S fertilization interact in the determination of the expression pattern of B-hordein genes. The transcription level of non fertilized (N0S0) and double fertilized (N1S1) plants was similar. Plants fertilized only with N (N1S0) showed an expression level lower than that of all the other treatments except for N0S1 at the last sampling date. This suggests that, at least under the S soil availability of our assay, it is not S availability but N/S ratio which is sensed by the plant to regulate the expression of these genes (Fig. 4A). On the contrary, C-hordeins reached a much higher level of expression in N fertilized plants and do not fluctuate upon S fertilization (Fig. 4B), indicating the regulation of these genes by plant N status as previously reported by Giese and Hopp (1984) and Qi et al. (2006).

Also in spike tissue, soluble protein concentration increased over time in S-fertilized plants, whereas it remained constant in non fertilized ones (Fig 4C). It has been shown that albumin and globulin (soluble grain protein fraction) content is positively correlated with the diastatic power (Wang et al., 2007), which in turn is closely related to β-amylase activity (Georg-Kraemer et al., 2001), an enzyme involved in starch degradation. Besides, malt diastatic power has been shown to increase in response to S fertilization in highly S-deficient soils (Zhao et al., 2006). Thus our findings, in accordance with the literature, suggest the importance of S fertilization for the generation of an adequate amount of grain soluble proteins that later would impact on malting quality.

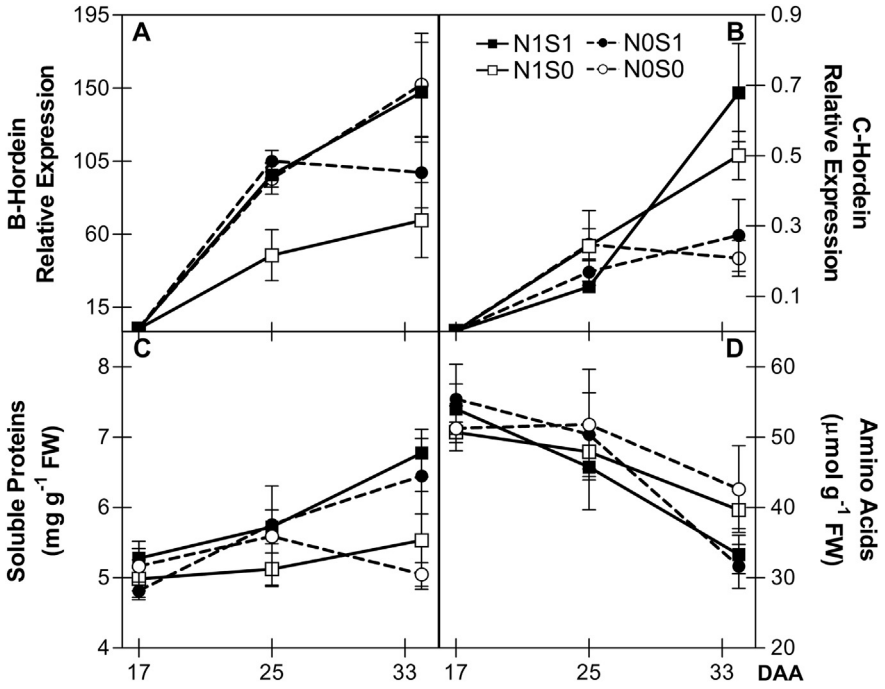

Fig. 4. Relative expression of (A) B-hordein and (B) C-hordein genes and concentration of (C) soluble proteins and (D) amino acids in spikes of N1S1 (—■—), N1S0 (—□—), N0S1 (- -●- -) and N0S0 (- -○- -) plants during grain filling. Data are the means ± SE ($n = 3$ for A and B; $n = 4$ for C and D). DAA = days after anthesis. *P*-values from the three-way ANOVA are shown in Table 1.

In turn, free amino acid concentration decreased in time but no significant differences were observed between treatments. However, a tendency to accumulate more amino acids in S non-fertilized plants at the end of the grain filling was found (Fig 4D), probably due to an accumulation of non-sulfur amino acids in the grain, such as asparagine which was observed to accumulate in wheat grain under S deficiency (Shewry et al., 2009).

Finally, soluble sugar concentration decreased during the grain filling period whereas starch concentration increased, but no differences between treatments were found (data not shown).

3.5. *Phloem sugars and amino acids as potential regulators of hordein expression*

The changes in the amino acid and sugar phloem export rate seem not to correlate with the concentration of both metabolites in the leaves, suggesting that the availability of each metabolite in leaves would not be a limiting factor to the establishment of the export rate in each treatment (Figs. 2B, C and 3). Besides, the amount of amino acids and sugars exported via phloem seems not to regulate the concentration of those metabolites in the spike tissue either (Figs. 3 and 4D). From these observations, it seems more likely that phloem amino acids are used for spike storage protein synthesis, since the expression level of B- and C-hordeins also fluctuated in relation to N and S availability (Fig. 4A and B). On the contrary, phloem sugars seem not to greatly contribute to starch synthesis, since the pattern of starch accumulation in the grain (data not shown) do not reflect the changes occurring in the sugar export rate (Fig. 3B). This is in agreement with Weichert et al. (2010) who observed no changes in starch concentration of wheat grains in response to an improvement of the sucrose uptake capacity due to the overexpression of a barley sucrose transporter (HvSUT1) gene.

Therefore, we decided to analyze the potential relationship between hordein gene expression and the rate of assimilates arriving to the spikes. The C- and B-hordein genes expression correlated with amino acid and sugar exudation rate respectively (Fig 5A and D). From this analysis, a curvilinear correlation between C-hordeins and the amino acid exudation rate was found at 25 DAA whereas a positive linear correlation was found at 34 DAA, suggesting a regulation of C-hordein expression by the rate of amino acids arriving to the spikes when the transcription level of these genes reaches to its peak of expression (Fig. 5A). This finding corroborates in field-grown plants the observation made in liquid culture spikes, that C-hordein expression is stimulated by the increment in the amino acid concentration in the medium (Bottacin et al., 1985). Bottacin et al. (1985) also reported that feeding with glutamine or cysteine to the spike did not increase the amount of S-rich B-hordeins, and in our field-grown plants, we neither found any correlation between B-hordeins and phloem amino acids (Fig. 5C).

Besides, in this assay, we did not find any correlation between the sugar exudation rate and C-hordein transcription level (Fig. 5B), but a positive linear correlation with B-hordeins was found (Fig. 5D). This result is also in agreement with Weichert et al. (2010) who observed an increment in the prolamine fraction of the wheat grains with an improvement of sucrose uptake. Given the well known role of sugars as signaling molecules and as regulators of gene expression (see Eveland and Jackson, 2012), it is possibly to presume a signaling role of phloem sugar to determine the expression level of B-hordein genes as a function of nutrient availability.

In conclusion, our results highlight phloem exudation rate of sugars and amino acids as potential regulatory intermediaries of B and C hordein gene expression level, which in turn, modulates the grain prolamine content and composition. Prolamines, as main component of the endosperm protein matrix, have been shown to affect several malting quality traits such as the endosperm modification during malting (Shewry et al., 2001) and the beta amylase activity (Qi et al., 2006). Therefore deepening knowledge of the

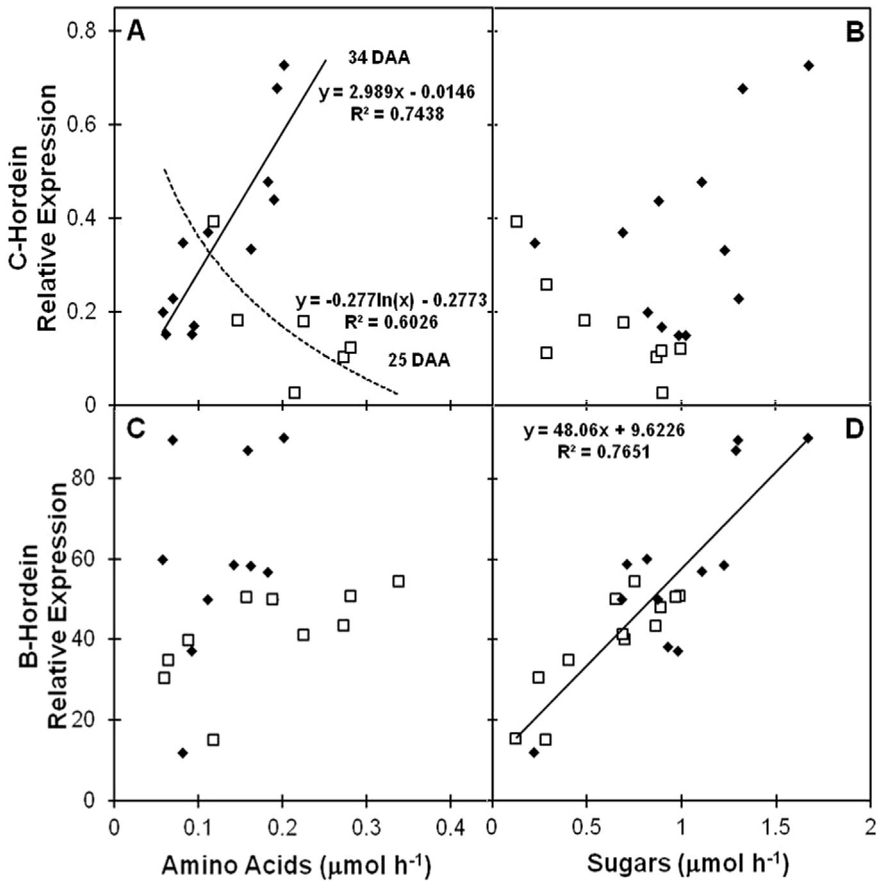

Fig. 5. Correlation between (A) C-hordein gene expression and amino acids phloem export rate, (B) C-hordein gene expression and sugars phloem export rate, (C) B-hordein gene expression and amino acids phloem export rate and (D) B-hordein gene expression and sugars phloem export rate. DAA = days after anthesis.

regulation of assimilates remobilization via phloem could contribute to the development of new strategies to improve the quality of barley grains for the malting industry.

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