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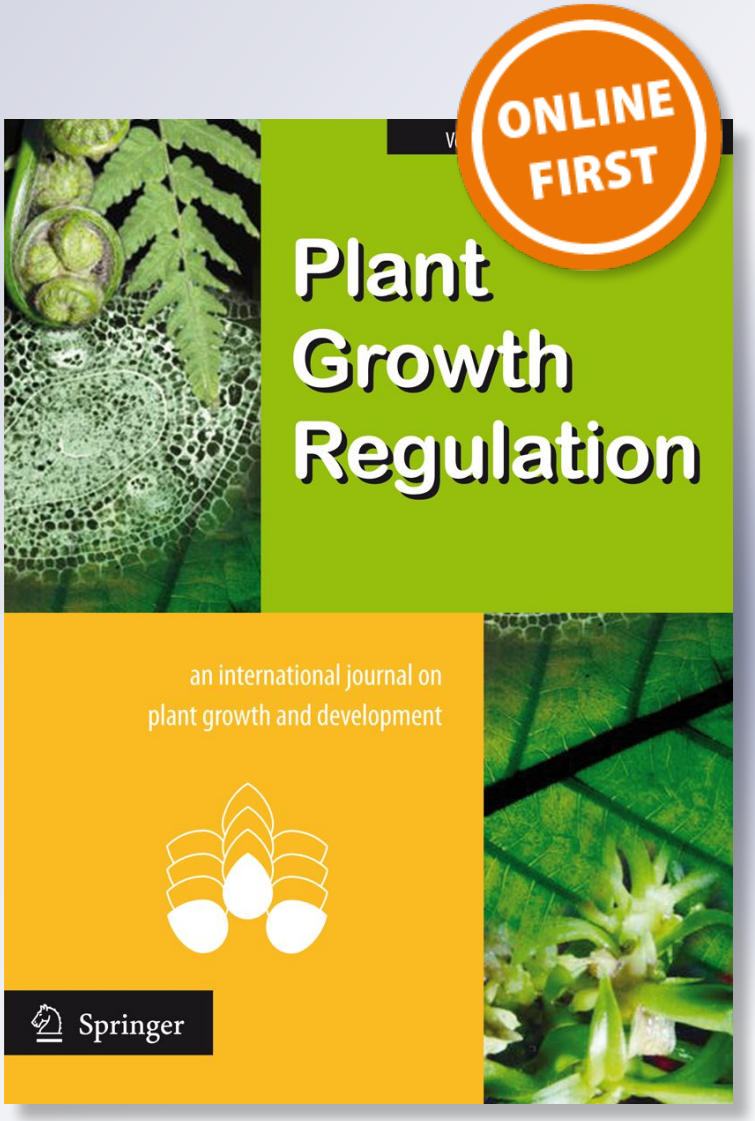

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ORIGINAL PAPER

Phloem transport of amino acids is differentially altered by phosphorus deficiency according to the nitrogen availability in young barley plants

Maria V. Criado[1] · Cintia G. Veliz[1] · Irma N. Roberts[1] · Carla Caputo[1]

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Abstract The phloem transport of amino acids is a key step in the efficient use of nitrogen (N). Despite the importance of this issue, little information is known about the regulation of phloem transport of amino acids in plants with low phosphorus (P) supply and even less in relation to N availability. To this end, we studied not only the assimilate partitioning in young barley plants grown with low N or/and P supply, but also we analyzed the implications of the different isoforms of glutamine synthetase, cytokinin oxidase/dehydrogenase 2 and several senescence-related proteases. Our results demonstrated that low P supply causes an accumulation of different nitrogenous compounds in expanded leaves depending on N availability and an inhibition of the phloem exudation rate of amino acids only in high-N plants, indicating an interaction between N and P in the establishment of N-partitioning. The accumulation of nitrogenous compounds in leaves of low-P plants without the accompaniment in amino acid export to the phloem was not related to an increase in nitrate assimilation pathway neither with the modulation of glutamine synthetase 1_1 expression, as it had been observed for N availability. But, these results could be explained as a consequence of a delay in the transition from sink to source of leaves, thus keeping the older leaves as sink organs, as indicated by the increase in cytokinin oxidase/dehydrogenase 2 expression and the repression of several senescence-related proteases in low-P plants with good availability of N.

✉ Carla Caputo
caputo@agro.uba.ar

[1] Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA)-CONICET, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

Keywords Phloem transport · Amino acids · Low phosphorus and nitrogen supply · Sink-source transition · Barley

Abbreviations

CKs	Cytokinins
CKX	Cytokinin oxidase/dehydrogenase
DW	Dry weight
GS	Glutamine synthetase
IPA	Isopentenyl adenosine
N	Nitrogen
NR	Nitrate reductase
P	Phosphorus

Introduction

Nitrogen (N) is the most important mineral nutrient for plants, and the form and concentration in soils are major factors determining plant productivity and crop yield. Besides, in certain cereals as malting barley *(Hordeum vulgare* L.) is also important achieve very specific grain protein content. Studies in different plants such as *Arabidopsis*, pea and barley demonstrated that the amount and composition of amino acids that arrive to developing organs via phloem is essential for both grain yield and quality (Veliz et al. 2014; Tegeder 2014; Gourieroux et al. 2016; Santiago and Tegeder 2016), and although the study of phloem regulation is complex, progress has been made in recent years. Tegeder (2014) has pointed out that amino acid transporters are crucial for improving N use efficiency. In that respect, the amino acid permease 8 (AAP8) in Arabidopsis (Santiago and Tegeder 2016) and AAP7 in barley (Veliz et al. in press) have been suggested to play an important role in source to sink partitioning of N, and that their function

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affects source leaf physiology and seed yield. On the other hand, phloem transport of amino acid has been shown to fluctuate both diurnally and seasonally (Caputo and Barneix 1999; Gourieroux et al. 2016) and that the principal form of translocated organic N is species specific being glutamine in barley (Winter et al. 1992), glutamate and glutamine in wheat (Simpson and Dalling 1981; Caputo and Barneix 1997), but arginine and glycine in grape berries (Gourieroux et al. 2016). Despite the importance of this issue and the progress made in this regard, information about the cellular and molecular details of N phloem transport and N partitioning between source and sink tissue is still fragmented. Thus, it becomes important to close existing gaps in the knowledge of whole-plant N partitioning since this process may well be important component of cereal N use efficiency (Distelfeld et al. 2014; Tegeder 2014).

Generally for most cereal crops including barley, the absorbed nitrate is reduced first to nitrite and then to ammonium, via nitrate reductase (NR) and nitrite reductase, respectively. Next, the ammonium is assimilated into organic N compounds via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. The fate of the synthesized amino acids will depend on development state of the leaf. Developing (young) leaves constitute significant net importers (sink) for all nutrients, which are utilized to build the organ's cellular and molecular components. After sink-source transition, leaves become net exporters (sources) of carbohydrates from photosynthesis, while the import (through the xylem) and the export (through the phloem) of mobile nutrients are in equilibrium (Fischer 2007). The onset of N remobilization and leaf senescence is associated to a decrease in net nutrient content and with a transition to net export of mobile compounds (Fischer 2007). During senescence, numerous senescence-associated proteases are up-regulated to enable the remobilization of nutrients, such as N, from senescent tissues to developing organs; however, they not only have a nutrient recycling function, but also are involved in the regulation of the senescence process (Roberts et al. 2012).

Cytokinins (CKs) are important plant growth regulators that control many plant developmental events such as leaf expansion, chloroplast formation, growth of lateral buds, sink-source relation and the delay of leaf senescence, among others (Mok and Mok 2001). Cytokinin oxidase/dehydrogenase (CKX) enzyme is the only enzyme that has been unequivocally shown to catalyze the catabolism of specific CKs and, therefore, regulates the levels of CKs. The enzyme is encoded by a multigene family of CKX genes and the expression of particular genes is known to be organ-specific (Massonneau et al. 2004; Werner et al. 2006). Particularly, in barley, *HvCKX1* transcripts were found in all organs tested, *HvCKX2* transcripts were detected in grains and young and mature leaves, and *HvCKX3* transcripts were detected in grains and young leaves (Galuszka et al. 2004). However, the biological role of each isoform and their regulation are far from clear.

As already mentioned, GS is a key enzyme for assimilation of NH_4^+ derived from primary or secondary sources, into glutamine GS, but it also plays a central role in N metabolism. There are two major isoforms of GS, GS1 in the cytosol and GS2 in plastids, which are assumed to play non-overlapping roles (Caputo et al. 2009). Besides, while a single gene encodes the plastidic isoform in plants, the cytosolic isoform is encoded by a multigene family (Goodall et al. 2013). The function of GS1 is not yet fully elucidated, but it has been found to influence diverse processes such as amino acid phloem loading (Bernard and Habash 2009; Caputo et al. 2009), senescence (Miflin and Habash 2002) and grain filling (Tabuchi et al. 2005; Martin et al. 2006). On the other hand, it is well-known that the major role of GS2 is the re-assimilation of the ammonium released during photorespiration (Wallsgrove et al. 1987).

After N, phosphorus (P) is the second most frequently limiting nutrient for plant growth. It is known that P deficiency results in alterations in the absorption and assimilation of N and in increments of amino acid concentrations in leaves and roots (Rufty et al. 1993; Huang et al. 2008); however, the way in which the deficiency of P and its interaction with N influences the regulation of internal translocation of N is far from clear. Therefore, the aim of this study was to obtain new insights into the regulation of amino acid transport via phloem and the partition of assimilates in response to low P or/and N supply in young barley plants. Our results not only provide information on the metabolic changes associated with phloem transport in response to low P supply and their interaction with N availability in young barley plants, but also analyses the possible causes of such metabolic changes, particularly the implications of the different isoforms of GS, CKX2 and various proteases associated with senescence on the regulation of the fate of amino acids in leaves.

Materials and methods

Plant material and growth conditions

Barley plants (*Hordeum vulgare* L. cv. Scarlett) were kept in a growth chamber (16 h light/8 h dark; irradiance of 300 µmol m^{-2} s^{-1}, temperature 25 °C light/18 °C dark) in 350-cm^3 pots, using vermiculite as substrate. Each pot containing five plants represented one independent biological replicate. Plants were watered twice a week with 50 ml per pot of Hoagland's nutrient solution (pH 6) (Hoagland and Arnon 1950) with modifications so as to obtain the

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combinatorial of high (H) or low (L) supply of P and N: HNHP (200 µM KH_2PO_4, 10 mM KNO_3), HNLP (50 µM KH_2PO_4, 10 mM KNO_3), LNHP (200 µM KH_2PO_4, 2 mM KNO_3) and LNLP plants (50 µM KH_2PO_4, 2 mM KNO_3). After 19 days, when the third leaf reached full expansion, chlorophyll content was estimated by measurement of the greenness index of the leaves using a SPAD-MINOLTA. Samples of shoot, developing leaves (sink leaves), last expanded leaves (source leaves) and their phloem exudates were collected. Shoots were oven-dried at 60 °C for 48 h, whereas leaves were stored in freezer at −80 °C until analysis. The collection of phloem exudates was carried out by the facilitated exudation technique (Caputo and Barneix 1997). The last expanded leaf of the five plants of each pot was cut and placed in 2 ml of a 20 mM EDTA solution (PH 6) for 15 min to avoid contamination with xylem or cellular fluid and then the leaves were transferred to a fresh EDTA solution for 3 h in the dark to avoid transpiration. Afterward, the exudation solution was stored at −20 °C for further analysis.

Biochemical analysis

Leaf tissue was homogenized (2.5 ml buffer g^{-1} fresh weight) with 50 mM Tris–HCl buffer pH 7.5 containing 1% (w/v) polyvinyP-olypyrrolidone (PVPP). The homogenates were centrifuged at 10,000×*g* for 20 min and 4 °C. The supernatant was used for the determination of soluble proteins (Bradford 1976), nitrates (Cataldo et al. 1975), free amino acids by the ninhydrin method (Yemm and Cocking 1955), soluble sugars by the anthrone method (Yemm and Willis 1954) and NR (EC 1 .6.6.1) activity (Lewis et al. 1982). In phloem exudates, free amino acids and soluble sugars were also determined by ninhydrin and anthrone methods.

Total RNA extraction, cDNA synthesis and quantitative PCR

TRIzol® Reagent (Ambion, by Life Technologies) was used for RNA extraction following the manufacturer's protocol. One microgram of DNAse treated total RNA was used for cDNA synthesis by reverse transcription with M-MLV Reverse Transcriptase (Promega) following the manufacturer's protocol. cDNA samples were used as templates to quantify the expression of target genes. Quantitative PCR analysis was carried out using gene-specific primers obtained as follows: GS1_1, GS1_2, GS2 (Goodall et al. 2013), SAG12, PAPAIN, SUBTILASE and CND41 (Parrott et al. 2010), CKX2 (Galuszka et al. 2004) and ACTIN (Hansen et al. 2007) as reference gene. The 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), and the comparative Ct (threshold cycles) method (DDCt) was applied for relative quantification of gene expression using the Stratagene Mx3000Pro thermocycler software®.

Statistical analysis

The results were analyzed using two-way Analysis of Variance (ANOVA). When N × P interaction was significant ($p < 0.05$), the post-hoc test of variance (Least Significant Difference or LSD) was used and different letters were assigned to differentiate groups significantly different. When N×P interaction resulted non-significant, the ANOVA result was shown inside each graphic.

Results

Shoot biomass

Low-N or/and P barley plants had a lower dry weight (DW) than those well supplied (HNHP plants). However, the effect of low P-supply was greater in high-N plants than in low-N ones, since the DW was reduced by 42% in HNLP plants compared to HNHP and 19% in LNLP plants compared to LNHP, reaching HNLP and LNLP plants the same biomass accumulation (Fig. 1). The fact that the low P-supply did not cause the same response according to the status of N plants evidences the interaction N×P obtained by statistical analysis. Finally, the low N and P availabilities imposed caused a clear deficiency status of each nutrient, but did not produce a severe limitation to growth.

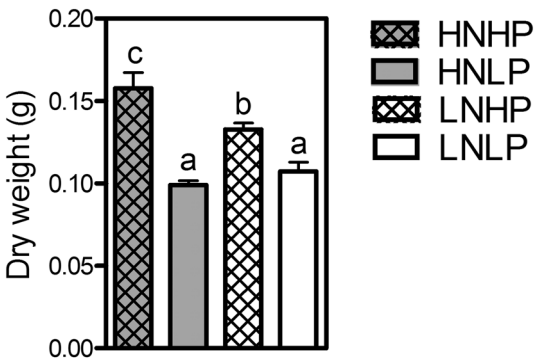

Fig. 1 Effect of low P-supply on shoot dry weight according to N availability in young barley plants. Plants were grown for 19 day in controlled conditions of temperature and irradiance, and watered with nutrient solution with modifications so as to obtain the combinatorial sufficient or insufficient supply of P and N: HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (*striped white bar*) and LNLP plants (*solid white bar*). Data are the means ± SE (n = 5). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

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Source leaves biochemical analysis

Low P-supply resulted in lower chlorophyll content in the source leaves of high-N plants (HNLP), but not of low-N ones (LNLP), showing an interaction between P and N. Besides, chlorophyll contents were lower in low-N and/or P plants compared with HPHN plants (Fig. 2a). Differently, effects of P and N, but not interaction, were observed for sugar concentration. That is, low-P plants had less sugar concentration than high-P ones, and low-N plants had higher sugar concentration than high-N ones (Fig. 2b). With regard to nitrogenous compounds, the effect of low P-supply changed depending on N availability, thus showing a N × P interaction. Low P-supply caused an increase in nitrate concentration only in high-N plants while this increase was not observed in low-N plants (Fig. 2c, N $p < 0.01$, P $p < 0.01$, N × P $p < 0.01$). Leaf amino acid concentration increased as a result of low p-supply and it decreased by low N-supply compared to HNHP plants; however, the effect of low P-supply was greater in high-N plants than in low-N ones (Fig. 2d, N $p < 0.01$, P $p < 0.01$, N × P $p = 0.03$). Finally, low P-supply provoked an increase in protein concentration in low-N plants, but no differences were found between HNHP and HNLP plants (Fig. 2e, N $p < 0.01$, P $p < 0.01$, N × P $p < 0.01$). In addition, low-N plants showed fewer nitrate, amino acid and protein concentrations than high-N ones (Fig. 2c–e).

Source leaves N assimilation

Regarding to NR activity, an interaction effect between N and P was observed. Low N and P-supply caused a decrease in NR activity relative to HNHP treatment; however, while low P-supply caused a decrease in high-N plants, it did not produce it in low-N plants (Fig. 3a). GS1_1, GS1_2 and GS2 gene expression showed different regulation in response to the low N and P supply (Fig. 3b–d). The relative expression of GS1_1 increased only as a result of low N-supply, showing higher relative expression in low-N plants compared to high-N ones (Fig. 3b). As to GS1_2, low-P plants showed higher relative expression than high-P ones, whereas low-N plants showed less expression that high-N ones (Fig. 3c). Finally, relative expression of GS2 decreased as a result of low N and P-supply; but the effect of low P-supply was higher in high-N plants than in low-N ones (Fig. 3d). The expression of GS isoforms allows distinguishing their different regulations, and a positive correlation between abundance and activity has been determined (Caputo et al. 2009).

Source leaves CKX2 and proteases

The relative expression of CKX2 was affected by the availability of P, but not by N; however the effect of P depended on N status, thus reflecting a N × P interaction. Namely, whereas low P-supply caused a decrease

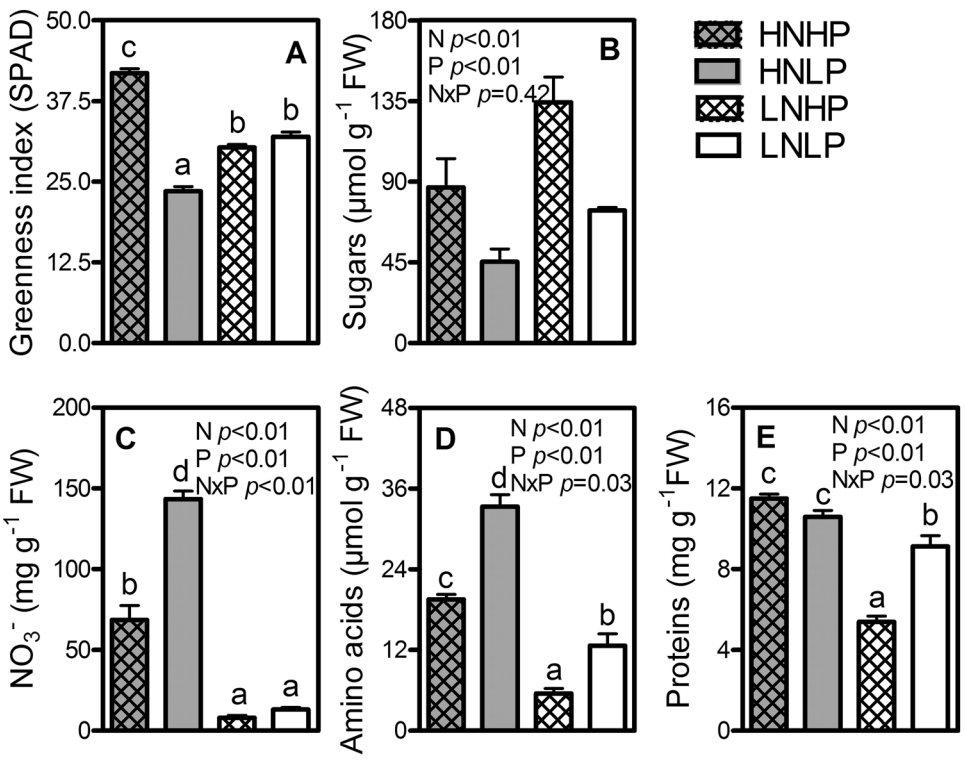

Fig. 2 Effect of low N and/or P supply on greenness index (**a**), soluble sugars (**b**), NO_3^- (**c**), free amino acids (**d**) and soluble proteins (**e**) in the source leaves of HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (striped *white bar*) and LNLP plants (*solid white bar*). Data are the means ± SE ($n = 5$). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

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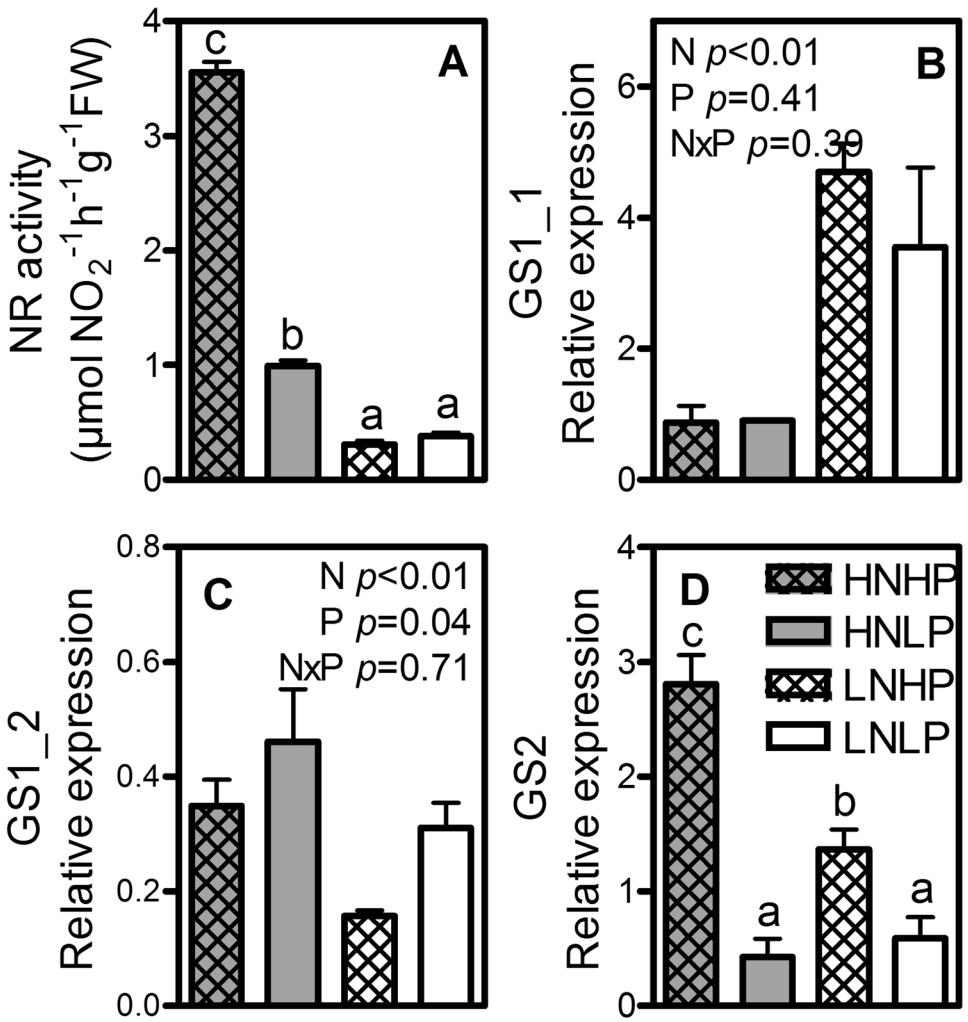

Fig. 3 N assimilation pathway and central role of GS in N metabolism: Effect of low N and/or P supply on NR activity (**a**) and relative expression of GS1_1 (**b**), GS1_2 (**c**) and GS2 (**d**) determined by qRT-PCR in real time in the source leaves of HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (*striped white bar*) and LNLP plants (*solid white bar*). The values were normalized according to actin expression. Data are the means ± SE ($n = 5$). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

in the relative expression of CKX2 in high-N plants, it showed no changes in low-N plants (Fig. 4a).

With respect to the relative expression of senescence-associated proteases SAG12, PAPAIN and SUBTILASE (Fig. 4b–d), interaction effects were found in all cases between N and P since both nutrients caused a decreased in the expression of these genes only in the high condition of the other nutrient (HNLP and LNHP relative to HNHP), while remained constant in SUBTILASE and caused an increased in SAG12 and PAPAIN, showing the same value that HNHP plants in the case of SAG12 and an intermediate one in PAPAIN, when the plants were deficient in the other nutrient. Conversely, low P-supply increased the relative expression of CND41 in high-N plants, whereas it decreased it in low-N ones, showing HNHP and LNLP plants the same values (Fig. 4e).

Phloem exudates

Both amino acid and sugar exudation rate decreased as a consequence of low N and P-supply and also a NxP interaction was observed (Fig. 5a, b). Plants with low P-supply showed lower amino acid exudation rate only when N was high, but not when N was low (Fig. 5a). Differently, low P-supply provoked a decreased in sugar exudation in both states of N plants, however the effect was higher in high-N plants than in low-N ones (Fig. 5b). Because no significant differences in the source leaf DW between treatments (data not shown), the rates of exudation of amino acids and sugars were expressed as nmol h^{-1} pot^{-1}.

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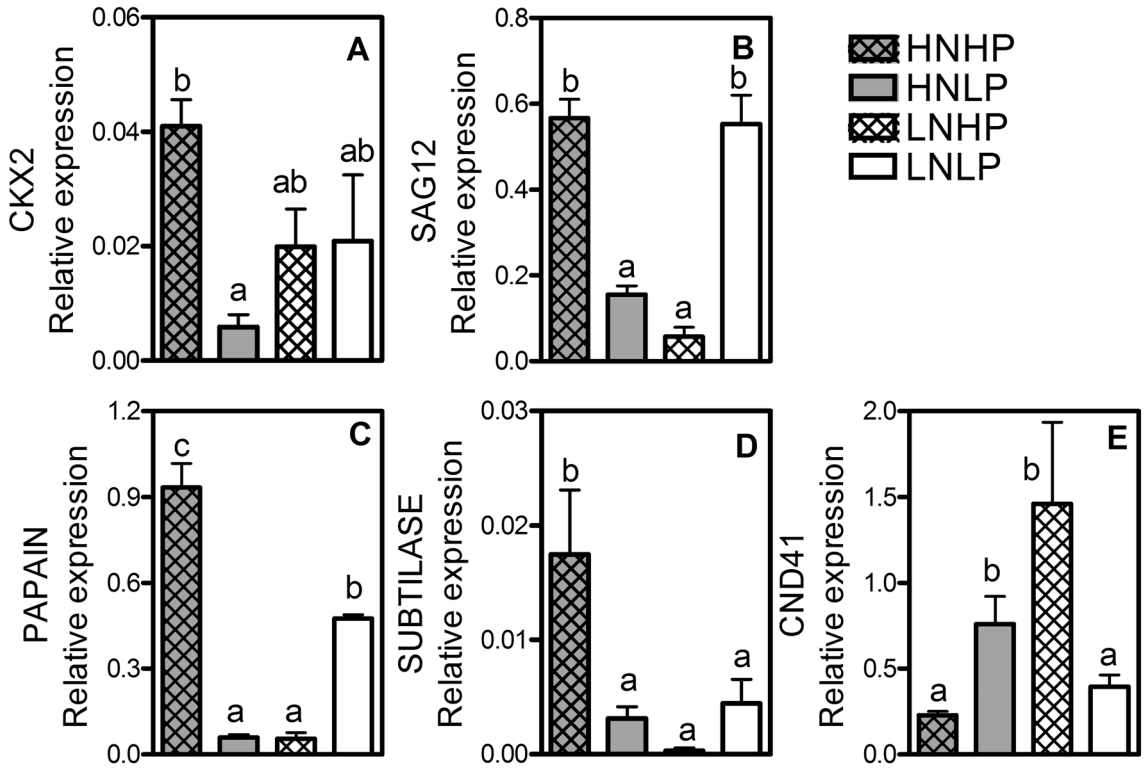

Fig. 4 Effect of low N and/or P supply on relative expression of CKX2 (**a**), SAG12 (**b**), PAPAIN (**c**), SUBTILASE (**d**) and CND41 (**e**) determined by qRT-PCR in real time in the source leaves of HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (striped *white bar*) and LNLP plants (*solid white bar*). The values were normalized according to actin expression. Data are the means ± SE ($n = 5$). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

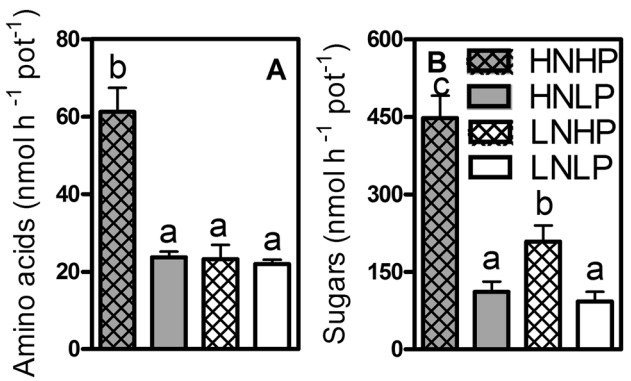

Fig. 5 Phloem exudation rate of amino acids (**a**) and sugars (**b**) obtained from 5 leaves of HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (*striped white bar*) and LNLP plants (*solid white bar*). Data are the means ± SE ($n = 5$). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

Sink leaves—biochemical analysis

The chlorophyll contents in the sink leaves of low-N and P plants were lower than HNHP ones, and also, a N × P interaction effect was observed as in source leaves. Specifically, low P-supply decreased the chlorophyll content in high-N plants, but did not change in low-N plants (Fig. 6a). Regarding sugar concentration, effects of N and P, but not N × P interaction were observed in the sink leaves as in the source ones. That is, whereas low P-supply caused a decrease in sugar concentration, low N-supply induced a slight increase (Fig. 6b). N, P and N × P effects were observed on nitrate concentrations in the sink leaves as in sources ones. Low P-supply increased nitrate concentration in high-N plants but not in low-N ones. Also, low-N plants showed fewer nitrates than HNHP plants (Fig. 6c). With regards to amino acid and protein concentrations, while N, P and N × P effects were observed in the source leaves, in the sink leaves only N effect was notice, being lower in LNHP and LNLP plants with respect to HNHP and HNLP ones (Fig. 6d, e).

Discussion

It is known that low P-supply produces alterations in the uptake and assimilation of N, resulting in increments of N concentration in leaves and roots (Rufty et al. 1993; Huang et al. 2008), but the effects of low P-supply and its interaction with N over phloem transport of amino acids is less

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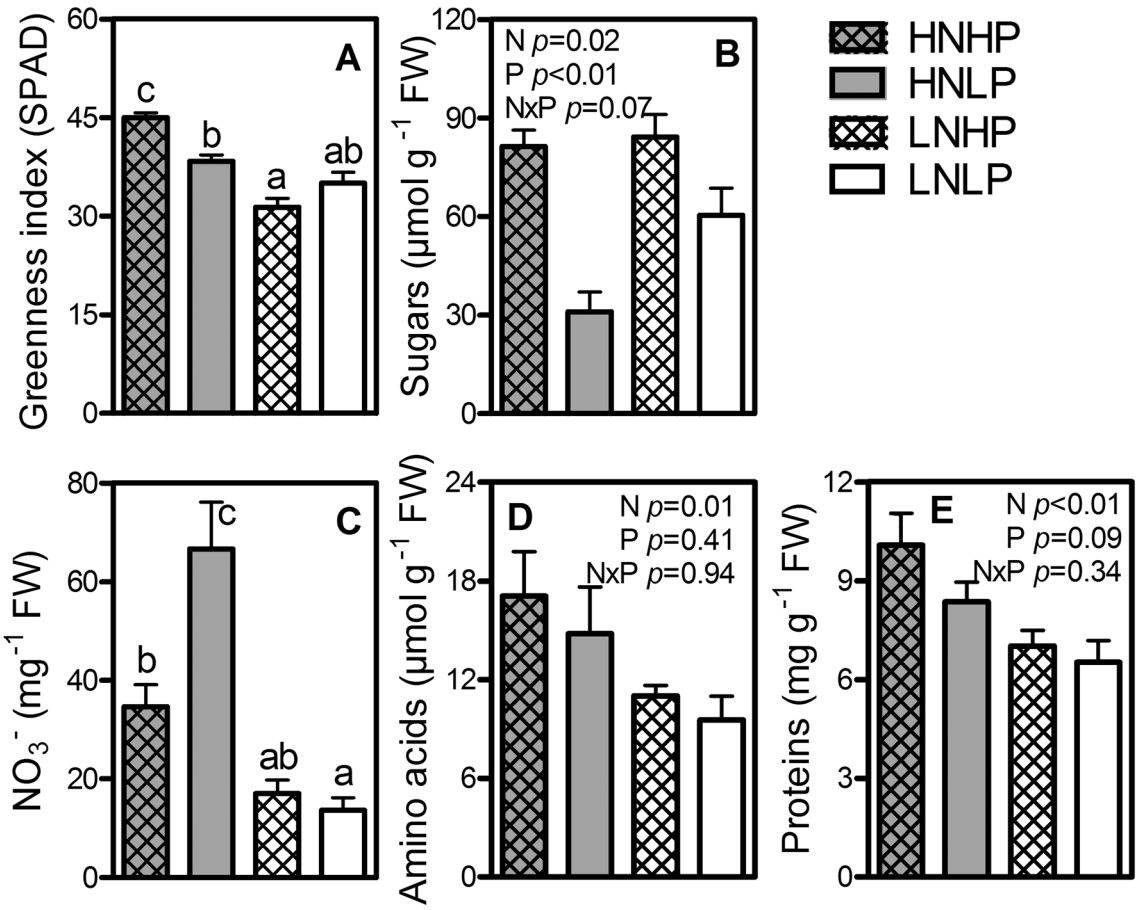

Fig. 6 Effect of low N and/or P supply on greenness index (**a**), soluble sugars (**b**), NO_3^- (**c**), free amino acids (**d**) and soluble proteins (**e**) in the sink leaves of HNHP (*striped gray bar*), HNLP (*solid gray bar*), LNHP (*striped white bar*) and LNLP plants (*solid white bar*). Data are the means ± SE ($n = 5$). Means with *different letters* are significantly different (LSD test at $p < 0.05$)

understood. The results obtained in this work show that the effect of low P-supply on the increment of nitrogenous compounds in the source leaf and the amino acid export via phloem depend on N availability. Low P-supply increased the nitrogenous compounds in source leaves such as nitrates and amino acids in high-N plants, and amino acids and proteins in low-N plants, but low P-supply decreased amino acid exports via phloem in high-N plants and produced no changes in low-N plants (Figs. 2, 5). In addition, low P-supply did not change protein and amino acid concentrations in the sink leaves, compared to the corresponding N status, but increased the concentration of nitrate in high-N plants (Fig. 6), suggesting that the xylem transport remains unchanged. On the other hand, the low supply of N, but not of P (LNHP plants) also produced a reduction in the phloem export rate of amino acids as it was previously reported (Caputo and Barneix 1997; Hammond and White 2008) but, unlike low P-supply, N also decreases the concentrations of nitrates, amino acids and proteins in both source and sink leaves compared to HNHP plants (Figs. 2, 5). The fact that amino acids are accumulated in the leaves without being exported to the phloem in low-P plants support the hypothesis that the rate of exudation of amino acids is not dependent on free amino acid concentration in leaves (Caputo and Barneix 1997). Besides, it is interesting to point out that no additive effect of N and P was observed in the reduction of the amino acid export via phloem. These results suggest that for plants to be able to export amino acids efficiently, it would be necessary not only a good availability of N, but also of P. Therefore, the supply of each nutrient separately would not be sufficient to induce amino acid export.

To establish if the increment of amino acids in expanded leaves (Fig. 2) is related to an induction of the nitrate assimilation pathway, NR activity and the expression of the different isoforms of GS were studied. The reduction of nitrate to ammonium is regulated by NR activity, being nitrate itself the primary positive regulator factor, but in addition to nitrate, NR is also regulated by a number of other factors, including light, growth, hormones, and reduced

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N metabolites (Sueyoshi et al. 1995; Stitt and Feil 1999). Accordingly, here we observed that NR activity decreased in plants that received low supply of nitrates (Fig. 3a). The effect of low P-supply and its interaction with N on the regulation of the activity of this enzyme is less well known, but even if the nitrate concentration was high, P deficiency also provoked a decrease in the NR activity, probably as a result of the increased amino acid concentration. In concordance, Wu et al. (2003) found that the gene encoding NR was repressed in leaves of Arabidopsis after 24 h of P starvation. But interestingly, GS1_2 expression increased (Fig. 3c), which would indicate an increase in ammonium assimilation. It was observed in barley that GS1_2 mRNA is localized in the leaf mesophyll cells, in the cortex and pericycle of roots being the dominant GS1 isoform in these tissues and that GS1_2 expression increases in leaves with an increasing supply of N, suggesting a role in the primary assimilation of N (Goodall et al. 2013). On the other hand, it is known that low P-supply decreases photosynthesis and carbon assimilation, and therefore, carbon-limiting conditions are generated (Wu et al. 2003; Huang et al. 2008). As barley is very sensitive to ammonium toxicity, a highly effective strategy for ammonium assimilation mechanism under the carbohydrate-limiting conditions caused by P would be the synthesis of glutamine and asparagine since they have a high C/N ratio as it has been pointed out by Huang et al. (2008). Regarding GS2 expression, it is well known that the major function of GS2 in chloroplasts is the reassimilation of ammonium released during photorespiration (Wallsgroove et al. 1987) and that low N-supply has a repressor effect over GS2 expression (Caputo et al. 2009) as it was seen here (Fig. 3d). Then, the down-regulation of GS2 expression in the leaves of low-P plants (Fig. 3d) could be explained by the known diminution of photosynthesis and photorespiration process under P deficiency (Wu et al. 2003; Huang et al. 2008).

GS plays a central role in N metabolism and this complex role varies according to the context in which the metabolism is taking place (Miflin and Habash 2002). Particularly, many studies have suggested that the cytosolic GS1 plays a central role in amino acid phloem loading given its location in the phloem and its modulation by N availability (Caputo et al. 2009). Furthermore, Goodall et al. (2013) reported in barley that GS1_1 mRNA is localized in vascular cells of different tissues, mainly in the stem and its expression changes in response to N supply. In this sense, our work showed that GS1_1 expression increased in low-N plants (Fig. 3a); but interestingly, also demonstrated that it did not vary with the availability of P, thus indicating that the expression of this gene would not be involved in the regulation of amino acid transport via phloem in response to P availability, differently to the response to N availability.

It is known that CKs retain the sink activity of the older leaves by inhibiting export of amino acids and sugars by the phloem, decreasing protein degradation and maintaining protein synthesis (Criado et al. 2009). Therefore, the non-exportation of the accumulated amino acids in sources leaves of low-P plants may be attributed to a delay in the leaf transition from sink to source state and consequently a delay in the onset of senescence. To test this hypothesis we measured the expression of HvCKX2, the principal enzyme that irreversibly degrades CKs in barley mature leaves (Galuszka et al. 2004), together with the expression of several proteases related to senescence as SAG12, PAPAIN and SUBTILASE and the expression of a protease non-related to senescence such as CDN41 as a control (Parrott et al. 2010; Roberts et al. 2012). Indeed, we found a decrease in the expression of HvCKX2 and the senescence-associated proteases in HNLP plants (Fig. 4a–d). As Galuszka et al. (2004) reported that transformation of the HvCKX2 gene into the tobacco genome unambiguously elevates the level of the endogenous CKX activity and causes phenotypic alterations typical for cytokinin-deficient plants, we postulate that the repression of CKX2 in HNLP plants leads to an increase in the concentration of CKs, which in turn, delay the onset of sink-source leaf transition and of the induction of the proteases involved in senescence. In that respect, Werner et al. (2006) reported that transcript abundance of AtCKX4, which is expressed in adult rosette leaves, lowered in response to P starvation. On the other hand, proteins encoded by the CKX family are known to differ in their biochemical properties, in particular the substrate affinity for the different cytokinins (Galuszka et al. 2007). Interestingly, isopentenyl adenosine (iPA) is both the best substrate for HvCKX2 (Galuszka et al. 2004) and the first signal that trigger senescence by N deficiency (Criado et al. 2007), suggesting that the onset of senescence would be triggered by the fall of iPA product of cleavage of CKX2, however it remains to be experimentally examined. In a different way, in low-N plants, low P-supply did not change CKs concentration in comparison to LNHP plants as indicated by CKX2 expression (Fig. 4a), suggesting that there is not a delay in the sink to source transition in expanded leaves. Besides, in LNLP plants the senescence process is advance compared to low N or P plants, as evidenced by the expression of senescence-associated proteases (Fig. 4b–d). This differential regulation remains an open question and, therefore, further studies are needed to provide new insights into the mechanism of accumulation of nitrogenous compounds in the leaves of plants with low N and P-supply.

Finally, very little is known about the effect of low P-supply on the export of sugars to the phloem. Cakmak et al. (1994) showed that P deficiency either had no effect or stimulated sucrose export in common bean. However, in our work,

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low P-supply decreased phloem exudation rate of sugars in both high and low-N plants (Fig. 5). There are considerable variation in the responses of shoot carbohydrate metabolism during P starvation, and some of this variation depend on the species, light intensity, leaf studied and treatment deficiency used (Hammond and White 2008) which could explain the differences in sugar levels in phloem, pointed out in the bibliography. The decay in this parameter observed here may be related with the suggested maintenance of source leaves as sink tissue under P deficiency by inhibiting CKs degradation, which was mentioned above to repress both sugar and amino acid transport via phloem (Criado et al. 2009). On the other hand, the observation that low P-supply did not caused a decrease in amino acid exportation rate in low-N plants, but it did it in the sugar exportation rate (Fig. 5), supports the hypothesis that the regulation of the export of amino acids and sugars are on separate ways, as it was pointed out before (Caputo and Barneix 1999) and it refuted the idea suggested previously that amino acid transport via phloem depends on the mass flow of sucrose driven by the active transport of sucrose involving phloem loading (Winter et al. 1992).

In summary, little information is known about the regulation of phloem export of amino acids in barley plants with low P-supply and even less in relation to N availability. Our results not only demonstrate that the accumulation of nitrogenous compounds in leaves of low-P plants is not accompanied by an increase in amino acid export to the phloem neither by an increase in nitrate assimilation, but also that the effects of low P-supply vary according to the N status of plants. In addition, we evidenced that both the increase in amino acid concentration in expanded leaves and the reduction in phloem exports, at least in high-N plants, might be explained as a consequence of a delay in the leaf transition from sink to source state, keeping the older leaves as sink organs, and bringing about a delay in the onset of senescence as indicated by the increase in CXK2 expression and the repression of several senescence-related proteases. Besides, there was no effect of P or NxP interaction on the relative expression of GS1_1, suggesting that the decrease in the export rate of amino acids by low P-supply is not related to the expression of this gene in contrast to what had been pointed out to the availability of N.

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References

Bernard SM, Habash DZ (2009) The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytol 182:608–620

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72:248–254

Cakmak I, Hengeler C, Marschner H (1994) Changes in phloem export of sucrose in leaves in response to phosphorus, potassium and magnesium deficiency in bean plants. J Exp Bot 45:1251–1257

Caputo C, Barneix AJ (1997) Export of amino acids to the phloem in relation to N supply in wheat. Physiol Plantarum 101:853–860

Caputo C, Barneix AJ (1999) The relationship between sugar and amino acid export to the phloem in young wheat plants. Ann Bot 84:33–38

Caputo C, Criado MV, Roberts IN, Gelso MA, Barneix AJ (2009) Regulation of glutamine synthetase 1 and amino acids transport in the phloem of young wheat plants. Plant Physiol Biochem 47:335–342

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6:71–80

Criado V, Roberts IN, Echeverria M, Barneix AJ (2007) Plant growth regulators and induction of leaf senescence in nitrogeN-deprived wheat plants. J Plant Growth Regul 26:301–307

Criado MV, Caputo C, Roberts IN, Castro MA, Barneix AJ (2009) CytokiniN-induced changes of nitrogen remobilization and chloroplast ultrastructure in wheat (*Triticum aestivum*). J Plant Physiol 166:1775–1785

Distelfeld A, Avni R, Fischer AM (2014) Senescence, nutrient remobilization, and yield in wheat and barley. J Exp Bot 65(14):3783–3798

Fischer AM (2007) Nutrient remobilization during leaf senescence. In: Gan S (eds) Senescence processes in plants, Blackwell, Sheffield, pp 87–107

Galuszka P, Frébortová J, Werner T, Yamada M, Strnad M, Schmülling T, Frébort I (2004) Cytokinin oxidase/dehydrogenase genes in barley and wheat. Eur J Biochem 271:3990–4002

Goodall AJ, Kumar P, Tobin AK (2013) Identification and expression analyses of cytosolic glutamine synthetase genes in barley (*Hordeum vulgare* L.). Plant Cell Physiol 54:492–505

Gourieroux AM, Holzapfel BP, Scollary GR, McCully ME, Canny MJ, Rogiers SY (2016) The amino acid distribution in rachis xylem sap and phloem exudate of *Vitis vinifera* 'Cabernet Sauvignon' bunches. Plant Physiol Biochem 105:45–54.

Hammond JP, White PJ (2008) Sucrose transport in the phloem: integrating root responses to phosphorus starvation. J Exp Bot 59:93–109

Hansen M, Lange M, Friis C, Dionisio G, Holm PB, Vincze E (2007) Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. J Exp Bot 58:3987–3995

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil, vol 347. 2nd edn. California Agricultural Experiment Station, Berkeley

Huang CY, Roessner U, Eickmeier I, Genc Y, Callahan DL, Shirley N, Langridge P, Bacic A (2008) Metabolite profiling reveals distinct changes in carbon and nitrogen metabolism in phosphate-deficient barley plants (*Hordeum vulgare* L.). Plant Cell Physiol 49:691–703

Lewis OAM, Watson EF, Hewitt EJ (1982) Determination of nitrate reductase activity in barley leaves and roots. Ann Bot 49:31–37

Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C, Dubois F, Balliau T, Valot B, Davanture M, Tercé-Laforgue T, Quilleré I, Coque M, Gallais A, Gonzalez-Moro M-B, Bethencourt L, Habash DZ, Lea PJ, Charcosset A, Perez P, Murigneux A, Sakakibara H, Edwards KJ, Hirel B (2006) Two cytosolic

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glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18:3252–3274

Massonneau A, Houba-Hérin N, Pethe C, Madzak C, Falque M, Mercy M, Kppecny D, Majira A, Rogowsky P, Laloue M (2004) Maize cytokinin oxidase genes: differential expression and cloning of two new cDNAs. J Exp Bot 55:2549–2557

Miflin BJ, Habash DZ (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J Exp Bot 53:979–987

Mok DW, Mok MC (2001) Cytokinin metabolism and action. Annu Rev Plant Biol 52:89–118

Parrott DL, Martin JM, Fischer AM (2010) Analysis of barley (*Hordeum vulgare*) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. New Phytol 187:313–331

Roberts IN, Caputo C, Criado MV, Funk C (2012) Senescence-associated proteases in plants. Physiol Plantarum 145:130–139.

Rufty TW, Israel DW, Volk RJ, Qiu J, Sa T (1993) Phosphate regulation of nitrate assimilation in soybean. J Exp Bot 44:879–891

Santiago JP, Tegeder M (2016) Connecting source with sink: the role of Arabidopsis AAP8 in phloem loading of amino acids. Plant Physiol 171:508–521

Simpson RJ, Dalling MJ (1981) Nitrogen redistribution during grain growth in wheat (*Triticum aestivum* L.). Planta 151:447–456

Stitt M, Feil R (1999) Lateral root frequency decreases when nitrate accumulates in tobacco transformants with low nitrate reductase activity: consequences for the regulation of biomass partitioning between shoots and root. Plant Soil 215:143–153

Sueyoshi K, Kleinhofs A, Warner RL (1995) Expression of NADH-specific and NAD (P) H-bispecific nitrate reductase genes in response to nitrate in barley. Plant Physiol 107:1303–1311

Tabuchi M, Sugiyama K, Ishiyama K, Inoue E, Sato T, Takahashi H, Yamaya T (2005) Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1; 1, a cytosolic glutamine synthetase1;1. Plant J 42:641–651

Tegeder M (2014) Transporters involved in source to sink partitioning of amino acids and ureides: opportunities for crop improvement. J Exp Bot 65:1865–1878

Veliz CG, Criado MV, Roberts IN, Echeverria M, Prystupa P, Prieto P, Gutierrez Boem FH, Caputo C (2014) Phloem sugars and amino acids as potential regulators of hordein expression in field grown malting barley (*Hordeum vulgare* L.). J Cereal Sci 60:433–439

Wallsgrove RM, Turner JC, Hall NP, Kendall AC, Bright SW (1987) Barley mutants lacking chloroplast glutamine synthetase-biochemical and genetic analysis. Plant Physiol 83:155–158

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T (2006) New insights into the biology of cytokinin degradation. Plant Biol 8:371–381

Winter H, Lohaus G, Heldt HW (1992) Phloem transport of amino acids in relation to their cytosolic levels in barley leaves. Plant Physiol 99:996–1004

Wu P, Ma L, Hou X, Wang M, Wu Y, Liu F, Deng XW (2003) Phosphate starvation triggers distinct alterations of genome expression in Arabidopsis roots and leaves. Plant Physiol 132:1260–1271

Yemm EW, Cocking EC (1955) The determination of amino-acids with ninhydrin. Analyst 80:209–213

Yemm EW, Willis AJ (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochem J 57:508–514