Developmental effects on ureide levels are mediated by tissue-specific regulation of allantoinase in *Phaseolus vulgaris* L.

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Abstract

The ureides allantoin and allantoate are key molecules in the transport and storage of nitrogen in ureide legumes. In shoots and leaves from *Phaseolus vulgaris* plants using symbiotically fixed nitrogen as the sole nitrogen source, ureide levels were roughly equivalent to those of nitrate-supported plants during the whole vegetative stage, but they exhibited a sudden increase at the onset of flowering. This rise in the level of ureides, mainly in the form of allantoate, was accompanied by increases in allantoinase gene expression and enzyme activity, consistent with developmental regulation of ureide levels mainly through the tissue-specific induction of allantoinase synthesis catalysed by allantoinase. Moreover, surprisingly high levels of ureides were also found in non-nodulated plants fertilized with nitrate, at both early and late developmental stages. The results suggest that remobilized N from lower leaves is probably involved in the sharp rise in ureides in shoots and leaves during early pod filling in N₂-fixing plants and in the significant amounts of ureides observed in non-nodulated plants.

Key words: Allantoin, allantoate, allantoinase, development, gene expression, nitrogen fixation, ureides.

Introduction

The ureides allantoin and allantoate are major forms of nitrogen transported from root nodules to shoots in tropical legumes. In these plants, nitrogen fixed by the rhizobia is used for purine synthesis. Through a series of enzymatic steps, purines are oxidized to allantoin and allantoate (Supplementary Fig. S1 available at JXB online). Ureides synthesized in the nodules are transported to the shoot where they should be degraded and their N content re-assimilated. *De novo* purine synthesis is the main route for ureide formation in nodules. However, purines involved in the biogenesis of ureides may also arise by turnover of nucleic acids (Zrenner et al., 2006).

Whatever the biosynthetic route, degradation of ureides starts with hydrolysis of the internal amide bond of allantoin, giving rise to allantoate, in a reaction catalysed by allantoin amidohydrolase [allantoinase (ALN); EC 3.5.2.5], which has been characterized in plants (Webb and Lindell, 1993; Yang and Han, 2004; Raso et al., 2007b). The pathway for degradation of allantoate into its end-products, glyoxylate and ammonia, is still under debate, since enzymatic activities have only been recently characterized in cell-free extracts, physiological studies are controversial, and the occurrence of several pathways for the degradation of both allantoate and ureidoglycolate has been reported (Todd et al., 2006; Muñoz et al., 2011). Nevertheless, most recent reports suggest that plants degrade allantoate via allantoin amidohydrolase (AAH; EC 3.5.3.9) and ureidoglycine amidohydrolase (EC 3.5.3.3–) (Todd and Polacco, 2004, 2006; Todd et al., 2006; Raso et al., 2007a; Werner et al., 2008, 2010, 2011; Serventi et al., 2010).

In soybean and other ureidic plants relying upon N₂ fixation as the sole nitrogen source, ureides may comprise up to 86% of the N in the xylem sap, whereas amino acids,
amides and nitrate are the major forms of nitrogen exported from the roots when plants are fertilized with nitrate (McClure and Israel, 1979; McClure et al., 1980). In these plants, it is assumed that ureides reach high concentrations only in nodulated, nitrogen-fixing plants, and determination of stem or petiole ureide levels has been established as an easy method to determine nitrogen fixation rates (McClure et al., 1980; Pate et al., 1980; Herridge, 1982; Patterson and LaRue, 1983; Herridge et al., 1990). Several reports have shown that plant development strongly influences the level of ureides in xylem sap and in leaves of uredic plants (Schubert, 1981; Herridge and Peoples, 1990; Aveline, 1995).

Changes in ureide levels upon plant development have been considered an important constraint for the use of the ureide assay as a convenient method to determine nitrogen fixation rates. The utilization of two different calibration curves for vegetative and reproductive growth phases has been proposed for soybean (Herridge and Peoples, 1990). Several reports have shown that nitrogen fertilization has negative effects on nodulation, nitrogen fixation, and the levels of ureides. This has led to the idea that high ureide concentrations are the direct consequence of nitrogen fixation (McClure et al., 1980; Atkins et al., 1982; Tajima et al., 2004). In contrast, upon nitrogen fertilization, uredic plants would use the amides asparagine and glutamine, instead of ureides, as their storage and exportable nitrogen (Pate et al., 1980; Streeter, 1985; Leidi and Rodriguez-Navarro, 2000).

Ureide levels have been shown to rise under water stress conditions, and it has been suggested that the accumulation of ureides may be responsible for the feedback inhibition of nitrogen fixation in these adverse situations (Serraj et al., 1999; Serraj, 2003; King and Purcell, 2005). Recent work has shown that ureide levels increase considerably in non-nodulated common bean plants suffering water stress. Recycling of nitrogen from proteins or nucleic acids in tissues undergoing drought-induced senescence was considered the possible source of these ureides (Alamillo et al., 2010). Nevertheless, the actual source of ureides and the molecular signalling leading to ureide accumulation under these conditions need to be clarified further.

Despite the great relevance of ureides, especially in legumes, there is little information on the regulation of genes and enzyme activities of ureide metabolism. Moreover, the scarce genomic information in uredic legumes has precluded the development of a broad-range expression analysis that could shed light on the regulation of these pathways. The recent availability of the fully sequenced genome of soybean (http://www.phytozome.net/soybean), as well as the ongoing projects for the sequencing of bean genomes, will help to compensate this lack of information, but, to date, there are only a few reports, focusing on a limited number of experimental conditions, in which regulation of ureide metabolism gene expression has been investigated in a uredic plant (Werner et al., 2008; Charlson et al., 2009; Alamillo et al., 2010; Yang et al., 2010).

In this work, an in-depth physiological and molecular analysis of ureide metabolism during development of nodulated and nitrate-fertilized, non-nodulated, P. vulgaris plants is presented. Under both regimes, ureide levels, nitrogen fixation rates, and amino acid and nitrate concentrations were measured, and the changes in the expression of genes and the activity of their encoded enzymes involved in ureide degradation during plant development were analysed.

The results presented here suggest that an increase in ureides at the beginning of the reproductive stage of development in Phaseolus vulgaris plants is mediated by the induction of ALN, and that a significant proportion of these ureides do not depend on nodule nitrogen fixation but instead originate from remobilization of nitrogen in the oldest vegetative tissues.

Materials and methods

Biological material and growth conditions

Phaseolus vulgaris L. cv. Great Northern seeds were surface sterilized by sequential dipping in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min), and washed thoroughly with distilled water. Soaked seeds were allowed to germinate on wet paper under sterile conditions. After germination, 3–4 seedlings were sown on each pot (16 cm diameter, 18 cm height) filled with an artificial substrate composed of vermiculite/perlite (2/1, w/w) and inoculated with 1 ml per plant of a fresh suspension of Rhizobium leguminosarum bv. phaseoli strain ISP 14, which had been cultured overnight at 28 °C to 0.8–1.0 (OD600 nm), corresponding to ~108 cells ml−1. Plants were cultured in a growth chamber under a 16 h light, 8 h dark photoperiod, with 200 μm2 s−1 lighting, 70% relative humidity, and 26–21 °C day–night temperatures. Inoculated plants were first watered with 5 mM KNO3 as a single, starter nutrition, and then three times a week with nitrogen-free nutrient solution (Rigaud and Puppo, 1975) during the whole of plant development, whereas the nitrate-fed, uninoculated, plants were watered with nutrient solution containing 10 mM KNO3.

Plant material collected at the indicated times after sowing was frozen with liquid nitrogen and stored at −80 °C until further analysis. Routinely, tissue samples were obtained as follows: the fourth trifoliate leaves; shoot tissue portions including the basal, middle, and apical stem after removal of leaves; and whole roots after careful removal of nodules were collected. In some experiments, the primary leaves and the uppermost, unfolded, leaves were also collected, and stored as independent leaf samples. Nodules collected from each individual plant were weighed and photographed, as a visual control of similar nitrogen fixation capacity among the different plants and experiments. Samples were collected from three independent experiments.

Nitrogen fixation

Nitrogenase (EC 1.7.9.92) activity was measured as the representational H2 evolution in an open-flow system (Witty and Minchin, 1998) using an electrochemical H2 sensor (Qubit System Inc., Canada). For nitrogenase activity measurements, nodulated roots from individual plants were sealed in 0.5 litre cylinders and H2 production was recorded according to the manufacturer’s instructions. The apparent nitrogenase activity (ANA; rate of H2 production in air) was determined under Ar:O2 (80%:20%) using a total flow of 0.4 l min−1. After reaching steady-state conditions, total nitrogenase activity (TNA) was determined under Ar:O2 (79%:21%) at a flow of 0.4 l min−1. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as 1–(ANA/TNA). Standards of high-purity H2 were used to
calibrate the detector. At least five replicates were measured in each experiment (biological replicates) for each condition.

Gene expression analysis

Total RNA was isolated from different tissues using the TRI REAGENT® RNA-DNA/Protein Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Prior to reverse transcription-PCR (RT-PCR), the RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) at 37 °C for 30 min. Lack of PCR amplification of the 18S rRNA was used to check the successful removal of DNA. First-strand cDNA synthesis was carried out using 2 μg of DNA-treated RNA using iScript™ reverse transcriptase (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) was carried out with the iCycler iQ System using the iQ SYBR-Green Supermix (Bio-Rad) and the gene-specific primers listed in Supplementary Table S1 at JXB online. The PCR program was as previously reported (Alamillo et al., 2010) and the amplification efficiency of the primers, calculated by serial dilutions of cDNA, was >90%. The ureide metabolism transcript levels were normalized with Actin-2 transcript expression as the reference gene, and were analysed using the method of Livak and Schmittgen (2001). All the reactions were set up in triplicate (three technical replicates) using three RNA preparations with plant tissues from three independent experiments (biological replicates).

Determination of enzymatic activities

All procedures for crude extract preparation were carried out at 0–4 °C. Frozen plant material was ground to a fine powder with liquid nitrogen. Plant extracts were obtained by adding 4 ml of extraction buffer per gram of tissue. The extraction buffer for each enzyme was the same as used in each of the assays, according to published reports. After 5–10 min of incubation, the resulting homogenate was centrifuged at 15 000 g for 5 min at 4 °C and the resulting supernatant was used as the crude extract. Uricase (UO) activity was measured by the decrease in absorbance at 292 nm due to the aerobic oxidation of urate in a reaction mixture containing 0.1 mM uric acid, in 0.1 M TRIS-HCl, pH 8.5, and an appropriate amount of crude extract (Pineda et al., 1984). ALN activity was determined basically by the procedure described in Raso et al. (2007b). Briefly, enzymatic production of allantoate was determined in a reaction mixture consisting of 50 mM TRIS-HCl, pH 7.8, 1 mM MnSO₄, 12 mM allantoin, and plant extract. The reaction was carried out at 35 °C and aliquots were taken at several time points to determine the allantoate concentration. Allantoate-degrading activity was determined following the production of ureidoglycogalate using a slight modification of the protocol described by Raso et al. (2007a). The reaction mixture was 50 mM triethanolamine-NaOH, pH 7.0, 1 mM MnSO₄, 6 mM potassium allantoate, 0.70 mM phenylhydrazine-HCl, and an appropriate amount of plant extract. The reaction was carried out at 35 °C and aliquots were taken at several time points to determine the ureidoglycogalate concentration. Controls were systematically used to account for the non-enzymatic decay of substrates. One unit (U) of enzymatic activity is the amount of enzyme that catalyses the transformation of 1 μmol of substrate per minute. Results of enzymatic activities are given as their specific activity (mU mg⁻¹ protein).

The results are expressed as means of the values from at least three independent experiments. Enzymatic assays and analytical determinations from each biological experiment were carried out in duplicate (technical replicate).

Xylem sap collection

Xylem sap was collected at 1 week intervals from 3- to 7-week-old nitrogen-fixing or nitrate-supplied plants and analysed for ureide, nitrate and amino acid contents. For xylem sap harvesting, the shoots were cut with a razor blade, just above the cotyledonary node (McClure and Israel, 1979). To avoid contamination, the cut surface was rinsed with sterile water, and the sap was collected for a period of 20 min per plant. Sap collection from all the plants within a sampling lasted ~2 h, and it was always carried out before midday. Xylem sap was kept on ice during the collection process and then immediately frozen until analysis.

Analytical determination

The concentration of ureides was determined by the colorimetric assay of glyoxylate derivatives as described by Vogels and Van der Drift (1970). In this assay, allantoin and allantoate are independently determined after their chemical transformation to glyoxylate. The values of total ureides in crude extracts are the sum of allantoin plus allantoate.

Individual free amino acids of the sap were assayed and quantified by reverse-phase HPLC of their OPA (o-phthalaldehyde) and FMOC (9-fluorenylmethoxy carbonyl chloride) derivatives using a Zorbax Eclipse AAA (4.6 mm×150 mm×5 μm) chromatography column and an Agilent 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). The chromatographic separation was carried out at 40 °C using a flow rate of 2 ml min⁻¹ according to the method described by Henderson et al. (2000). For detection, a diode array detector at 338 nm was used for OPA-amino acids and 262 nm for FMOC-amino acids; and a fluorescence detector (340 nm excitation and 450 nm emission from 0 min to 15 min, and excitation at 266 nm and emission at 305 nm from 15 min to 26 min). Peak identification was performed using the 24 amino acids standard mix as described by Henderson et al. (2000).

Nitrate in the xylem sap was determined according to Cataldo et al. (1975).

Soluble protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a protein standard.

The results are expressed as means of the values from at least three independent experiments. Determinations from each biological experiment were carried out in duplicate (technical replicate).

Results

Developmental stage regulates ureide levels in common bean tissues

To study the effects of development on the regulation of ureide metabolism, the levels of ureides were first determined in tissues of P. vulgaris depending on N₂ fixation as the sole nitrogen source or in plants fed with nitrate, during both the vegetative and reproductive phases of development. For that, plants were cultured under either condition, as described in the Materials and methods, and tissues were collected at 21, 28, 35, 42, and 49 days after sowing (DAS). At these times, plants were at the vegetative stage at 21 d and 28 d, and most of them have already flowered at 35 d, whereas samples at 42 d and 49 d correspond to pod development and early seed-filling stages (Table 1).

The ureides allantoin and allantoate were determined in roots, shoots, and leaves from the tissues collected at the different times after sowing. The ureide concentration increased during development in roots, shoots, and leaves from nitrogen-fixing plants and it was higher in most tissues
from nitrogen-fixing plants than in the samples from the plants fertilized with nitrate (Fig. 1). It was noticeable that accumulation of ureides in nodulated plants was mostly in the form of allantoin in leaves and stems, but it was equally distributed between allantoin and allantoate in roots. In shoots, similar amounts of both ureides were found at 21 d, but allantoin was more abundant than allantoate from 28 d onward, at it reached its maximum levels at the reproductive stage of development (35, 42, or 49 DAS) (Fig. 1). In roots, the concentration of ureides increased ~2.0- to 2.5-fold from 21 to 28 and 35 DAS. This increase in ureide levels was only observed in the nitrogen-fixing plants, whereas in the nitrate-fed plants the lowest level of ureides was found in roots collected at 35 DAS (Fig. 1A). In contrast to the moderate (2- to 3-fold) increase in ureide levels found in roots, stems and leaves from nitrogen-fixing plants showed a sharp increase in the level of ureides at 35 DAS. The concentration of ureides was maintained at a high level from 35 d to 49 d in shoots (Fig. 1B), whereas in leaves the ureide level showed a clear peak at 35 d and a rapid decline in subsequent weeks (Fig. 1C). As for roots, stems and leaves from nitrate-fed plants showed the lowest level of ureides at 35 d, but contained significant ureide concentrations, at both early (21 d) and late developmental stages (49 d). Interestingly, the level of ureides at 21 d was slightly higher in all tissues from nitrate-fed-plants than in the equivalent tissues from plants relying on nitrogen fixation as the sole nitrogen source (Fig. 1).

Nitrogen fixation rates during the development of P. vulgaris

Nitrogen fixation was measured as the ANA and TNA, and the EAC was also estimated (Witty and Minchin, 1998). The maximum nitrogen fixation estimated during common bean development was found at 28 DAS (Fig. 2A; Supplementary Table S2 at JXB online). Moreover, nodule fresh weight (Fig. 2B), uricase activity (UO), and ureide content were measured in nodules from P. vulgaris roots collected at different times during plant development. As expected, uricase, a late nodulin with a key activity involved in ureide synthesis (Sanchez et al., 1987), showed a pattern of activity similar to that exhibited by nitrogen fixation (Fig. 2C), and similar kinetics were also obtained for the ureide content in these nodules (Fig. 2D). In contrast, maximum nodule fresh weight was slightly delayed with respect to nitrogen fixation (Fig. 2B), probably because at the times of highest nodule number (35–42 d) a significant part of these nodules might be already entering into senescence.

Developmental stage regulates allantoinase transcript levels

To get a better understanding of how changes in the ureide concentration in P. vulgaris tissues are regulated by the developmental stage, the expression level of genes involved in either allantoate synthesis or degradation was determined. For that, qRT-PCR was carried out using gene-specific primers for PvALN and PvAAH, and cDNAs derived from RNAs isolated from roots, stems, and leaves collected at several different times during plant development.

The expression level of ureide metabolism genes was normalized with respect to expression of Actin-2, used as internal control, and the cDNA from root samples at 21 DAS was used as the reference in each condition. A single-copy gene codes for AAH in P. vulgaris (Diaz-Leal et al., personal communication; EF650088), whereas two highly similar sequences coding for possible ALN genes have been found in the P. vulgaris genome (Duran and Todd, 2012; this study). The full-length clones have been obtained and their sequences have been deposited in the NCBI data bank (JQ282796 and JQ277455). However, only one of these two genes (PvALN 1) showed significant expression in P. vulgaris tissues analysed in this work, whereas basal levels of expression were found for the second ALN-coding gene (PvALN 2). The expression pattern shown in Fig. 3A does, therefore, resemble the expression of PvALN 1, although it was obtained with a primer pair potentially able to determine the expression of both genes (Supplementary Fig. S2 at JXB online). A comparison of expression of both ALN genes showing the low levels of PvALN 2 mRNA is presented in Supplementary Fig. S3. Expression of PvALN was higher in the stems than in the roots or leaves from N2-fixing plants. Moreover, a strong induction of PvALN transcript with growth time was shown in shoots from nitrogen-fixing plants. In contrast, PvALN mRNA did not show any significant changes in roots and it was only slightly induced in the leaves, with an expression pattern with time that resembles the nitrogen fixation

### Table 1. Growth parameters of P. vulgaris plants grown under N2 fixation or fertilized with nitrate

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<th>Days</th>
<th>N2 fixation</th>
<th>Nitrate</th>
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<tr>
<td></td>
<td>Growth (nodes)</td>
<td>Flowers (%)</td>
</tr>
<tr>
<td>21</td>
<td>V5±2</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>V6±2</td>
<td>15</td>
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<tr>
<td>35</td>
<td>V7±3</td>
<td>84</td>
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<td>42</td>
<td>V8±3</td>
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<td>49</td>
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activity of these plants (Fig. 3A). Surprisingly, the expression of ALN was similar or even higher in roots and leaves from plants fed with nitrate compared with the expression in nitrogen-fixing plants, although it did not show any large changes during vegetative development. Unlike roots and leaves, ALN expression was lower in stems from nitrate-fertilized plants than in the nitrogen-fixing plants, and it was induced only at the late developmental stage (42 DAS samples) (Fig. 3A).

In contrast to \textit{PvALN}, \textit{PvAAH} transcript expression (Fig. 3B) was higher in the leaves than in roots or shoots and, in general, its expression increased slightly along with the nitrogen fixation rates in shoots and leaves from nodulated plants. \textit{PvAAH} expression in roots from nitrogen-fixing plants did not change significantly, except for a moderate induction at late development stages. As already mentioned for \textit{PvALN} transcript expression, the \textit{PvAAH} expression level was similar, or even higher, in samples from nitrate-fed plants than in the same tissue from nitrogen-fixing plants; in roots and stems, there were only small differences between both conditions, except for the significantly lower expression found at 35 d in shoots from nitrate-fed plants compared with the nodulated plants, while in leaves \textit{PvAAH} reached even higher expression levels in the samples from nitrate-fed plants than in those from the nodulated plants (Fig. 3B).

It was remarkable that the changes in the expression level of \textit{PvAAH} during development were quantitatively lower (<2-fold induction) than those of the ALN gene in shoots from nitrogen-fixing plants, that reached values ~10 times higher in the latest growth stages than early in development (Fig. 3A, B). Besides the small changes in its expression levels, relative to the expression in 21-day-old roots, the expression of \textit{PvAAH}, normalized according to the internal control \textit{Actin-2}, was in general several fold lower than the

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**Fig. 1.** Ureide concentration during plant development in \textit{P. vulgaris} tissues. Crude extracts were obtained from frozen tissues collected at several time points during common bean development. Concentrations of allantoin (grey bars) and allantoate (black bars) in roots (A), stems (B), and trifoliate leaves (C) are shown. Results are the mean ± SD of four independent experiments.

**Fig. 2.** Developmental effects in N\textsubscript{2} fixation, nodule mass, uricase (UO) activity, and ureide contents in nodules from \textit{P. vulgaris}. Pattern of (A) apparent nitrogenase activity (ANA), (B) total nodule mass, (C) UO activity, and (D) ureide content in nodules from \textit{P. vulgaris} sampled during plant development.
expression of \(PvALN\) 1 (results not shown). In the nodules, the relative expression of \(PvALN\) 1, \(PvALN\) 2, \(PvAAH\), and the nodulin uricase \((PvUO)\) presented a similar trend (see Supplementary Fig. S4 at *JXB* online). The four genes showed the pattern of expression expected according to the kinetics of nitrogen fixation, with their highest expression levels at 21–28 d, thus preceding the maximum of nitrogen fixation activity.

**Developmental effects on the activities of the enzymes of ureide metabolism**

Enzymatic activities of ALN and AAH were measured in the plant tissues used previously to determine gene expression and ureide levels (Fig. 4). In stems and leaves, the specific ALN activity was \(\sim\)10 times higher than the AAH activity, whereas in roots ALN activity was only 2–3 times higher than that of AAH (Fig. 4).

ALN activity was higher in tissues from nitrogen-fixing plants than in plants grown on nitrate, except for samples at early times of development (21 d) in which lower ALN activity was found in roots and shoots from nitrogen-fixing plants compared with the plants grown on nitrate. The highest ALN activity was found in stems, with \(\sim\)3- to 5-fold the activity found in roots or leaves. Noticeably, the temporal evolution of activity in all analysed tissues was similar to the pattern of the ureide levels, although the only tissue showing strong increases in ALN-specific activity was the shoot (Fig. 4A).

In contrast to the effect of plant growth on ALN, developmental stage did not exert any significant effect on AAH activity, apart from a slight increase in activity at late times of development in roots. The highest level of specific

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**Fig. 3.** Analysis of relative transcript expression of allantoinase (ALN) and allantoate amidohydrolase (AAH) during development of *Phaseolus vulgaris* plants grown under nitrogen fixation or nitrate-fertilized conditions. Relative transcript expression of ureide metabolism genes analysed by qRT-PCR during plant development in tissues from common bean cultivated either under nitrogen fixation conditions or fertilized with nitrate. Data were normalized to the expression of the Actin-2 gene and are expressed relative to the expression of the corresponding gene in roots at 21 d, using the \(2^{-\Delta\DeltaCT}\) formula (Livak and Schmittgen, 2001). (A) Relative transcript expression of \(PvALN\) in roots, stems, and leaves at 21, 28, 35, and 42 DAS. (B) Relative \(PvAAH\) transcript in roots, stems, and leaves at 21, 28, 35, and 42 DAS.

**Fig. 4.** Activity of ureide metabolism enzymes in roots, stems, and leaves during development of *P. vulgaris*. Crude extracts were prepared from frozen tissues from nodulated plants grown under nitrogen fixation conditions (white bars) and non-nodulated, nitrate-fertilized plants (black bars). (A) Allantoinase activity. (B) Allantoate amidohydrolase activity. Tissue samples were collected at 21, 28, 35, 42, and 49 days after sowing (DAS). The enzymatic activities are represented as mU per mg of total soluble protein. Results are the mean ± SD of three independent experiments and each assay was performed in duplicate (technical replicate).
activity was found in roots, it was ~3 times lower in shoots, and the lowest level of activity was found in leaves (Fig. 4B). The high specific AAH activity in roots was most probably due to the low protein level of this tissue, since low activity levels were found for all the tissues analysed when expressed as activity per fresh tissue weight. Moreover, there were no clear differences between samples from nitrogen-fixing plants and those from nitrate-fed plants (Fig. 4B).

Young developing leaves accumulate large amounts of ureides, even in the absence of nitrogen fixation

The results presented in Fig. 1 showed that plant age strongly influenced ureide levels in trifoliate leaves. To check whether development exerts the same effect in old and young leaves co-existing within a plant, ureide content and ALN activity were determined in the oldest, primary leaves, and in the uppermost, still unfolded youngest leaves, from plants grown either under nitrogen fixation conditions or with nitrate as the main nitrogen source (Fig. 5). In the primary leaves, low concentrations of ureides were found in samples collected at 21–42 DAS, both in plants relying on nitrogen fixation and in nitrate-fed plants (Fig. 5A, B). In contrast, in the uppermost, youngest leaves, the level of ureides showed a sharp peak at 35 DAS in nitrogen-fixing plants (Fig. 5A), and a gradual increase upon development in the plants fed with nitrate (Fig. 5B). Moreover, the concentration of ureides was higher in the uppermost, unfolded leaves than in the mature trifoliate leaves analysed in Fig. 1, although they showed similar patterns during plant development. Surprisingly, in the youngest leaves from nitrate-grown plants, the ureides reached concentrations as high as those in the equivalent leaves from nodulated, nitrogen-fixing plants (Fig. 5A, B).

As well as the ureides, the ALN activity measured in the same samples was lower in the primary leaves, at any developmental stage, than in the uppermost, youngest, leaves. In general, similar levels of enzyme activity were found in the nodulated and in the non-nodulated plants (Fig. 5C, D). ALN activity showed a gradual, but not very high, increment with plant age in the primary leaves from plants grown under nitrogen fixation and it was maintained at constant levels in the plants fertilized with nitrate. In contrast, in the youngest leaves, ALN activity exhibited a similar pattern in both sets of plants. As previously stated, the activity was ~2–3 times higher in the uppermost, youngest, leaves than in oldest, primary leaves (Fig. 5C, D).

Changes in amino acid, ureide, and nitrate levels during development in xylem sap from nitrogen-fixing and nitrate-fed plants

Amino acids, ureide, and nitrate contents in the xylem sap from common bean plants were analysed in samples collected at several different times during plant development, both in nodulated and in non-nodulated, nitrate-fed plants. Xylem sap collection was carried out during the morning to avoid diurnal effects on transpiration, and each sample comprised the xylem saps collected during 20 min from four plants in each condition. Sample aliquots were used to measure the ureide, amino acid, and nitrate content within the same sample. The individual amino acid concentration for up to 18 amino acids was determined by HPLC analysis after their derivatization and calibration with appropriate standard curves. A summary of the results, showing total amino acid, ureide, and nitrate content in common bean xylem saps measured at 21, 28, 35, 42, and 49 DAS, is given in Table 2, whereas the concentration of the four most prominent amino acids, as well as contents of allantoin and allantoate, is depicted in Fig. 6. The profile for total soluble amino acids is also presented in Supplementary Fig. S5 at JXB online.

Under N₂ fixation, the amides asparagine and glutamine were the most abundant amino acids, followed by aspartate and glutamate. Aspartate and asparagine followed a similar pattern, with a higher level at 21 d, lower at 28 d and 35 d, and a sharp increase at 42 d, whereas the levels of glutamate and glutamine showed a steady increase during development, also peaking at 42 d (Fig. 6A). Interestingly, levels of glutamine (Fig. 6A) closely resembled the pattern of ureide accumulation (Fig. 6C). In contrast, increments in ureide levels at 28 and 35 DAS (Fig. 6C) coincided with the lowest levels of asparagine in these plants (Fig. 6A). In plants fertilized with nitrate (Fig. 6B), asparagine was the most abundant organic nitrogen compound, followed by aspartate, glutamate, and glutamine. In these plants, the asparagine levels increased during development, showing a peak at 42 d, reaching concentrations that were ~8 times the concentration of glutamate, glutamate, or even aspartate.

In N₂-fixing plants, the increment in xylem ureide correlates with increases in nodule fresh weight and matched the nitrogen fixation rates (Fig. 2) during the vegetative growth phase, but sharply increased after flowering (35–42 d) (Fig. 6C). The contents of allantoin and allantoate followed a similar pattern during plant growth, although allantonic acid was the major component of the ureide fraction at any of the developmental stages. Interestingly, significant levels of ureides were also found in the xylem from nitrate-fed plants, in which, at 42 d, ureides reached values that were ~40% of the concentration found in the N₂-fixing plants (Fig. 6C). Levels of both amino acids and ureides were lower at 49 DAS than at 42 DAS, suggesting that sources of organic nitrogen, either by fixation or assimilation, begin to be less operative.

As expected, the nitrate concentration was maintained at low levels in xylem sap collected from nodulated plants, but it reached concentrations that were 2–4 times higher than the 10 mM used in the nutrient solution in the plants fertilized with nitrate (Table 2).

Ureide, amino acid, and nitrate contents were used to determine the relative ureide nitrogen (RUN) content during development in common bean plants, according to Herridge (1982). RUN reached 91–93% at the times of the highest nitrogen fixation rates (28 d and 35 d) in the nodulated plants, and slightly decreased at later developmental stages. In contrast, in nitrate-fertilized plants, RUN
was \(\sim10\%\) during vegetative development, but rose to 16–17\% at the late, reproductive phase of development (Table 2).

**Discussion**

Many reports have shown a direct correlation between ureide levels and nitrogen fixation, although large amounts of ureide have also been observed in uninoculated plants and in non-nitrogen-fixing species, challenging the frequently held idea that significant ureide levels are only produced by nitrogen fixation in ureidic plants (Thomas et al., 1979, 1980; Brychkova et al., 2008; Santos et al., 2009).

The results presented in this work demonstrate that in *P. vulgaris* the concentration of ureides only shows a good correlation with nitrogen fixation until the onset of flowering. Afterwards, the amount of ureides in leaves and stems increases more than nitrogen fixation (Figs 1, 2). Moreover, leaves and stems from uninoculated, nitrate-fertilized plants also show significant ureide levels during their late development (Figs 1, 5; Table 2). The amount of ureides in uninoculated plants is comparable with the apparent excess of ureides in relation to fixation rates found...
after flowering in the nodulated, nitrogen-fixing plants, suggesting that they probably originated from the same source. The results presented in Fig. 5 strongly support this hypothesis, and point to remobilization of nitrogen from the oldest leaves as the main source for ureide synthesis and accumulation in shoots and developing tissues in the non-nodulated plants, and for the sharp increase in ureides during early pod filling in the nodulated plants when nitrogen fixation starts to decline (Figs 1, 2).

Changes in the correlation between nitrogen fixation and ureide levels with transition to reproductive development have been clearly demonstrated (Herridge and Peoples, 1990). Furthermore, early nitrogen remobilization in senescent tissues in an early maturing cultivar of soybean was reported as a possible source of error in the estimation of nitrogen fixation by the ureide assay method (Aveline, 1995). In fact, it has recently been shown that, under conditions of water stress, \textit{P. vulgaris} plants accumulate ureides even if they lack root nodules (grown with nitrate), and major accumulation of ureides occurs after drought has completely inhibited the N\textsubscript{2} fixation in nodulated plants (Alamillo \textit{et al.}, 2010). Remobilization of nitrogen from drought-induced senescent tissues was suggested as the most likely alternative source of ureides under such conditions.

Fischinger \textit{et al.} (2006) showed that nitrogen re-translocation from senescing lower leaves to common bean root nodules might be involved in the N-feedback regulation of nitrogen fixation. Moreover, ureide accumulation upon drought stress has been hypothesized to be responsible for N\textsubscript{2} fixation inhibition (Serraj \textit{et al.}, 1999). Therefore, it would be interesting to analyse further the actual contribution of ureides originating from senescing lower leaves to the decline in nodule activity shown at late developmental stages (Fig. 2).

The present results resemble those from early studies showing increases in ureide-N at flowering and early pod formation in non-nodulated \textit{P. vulgaris} L. (bushbean) (Thomas \textit{et al.}, 1979, 1980). It has been shown that the increase in ureides correlated with a peak in the specific activity of ALN in soybean (Thomas and Schrader, 1981). However, in these early reports, lack of genetic information precluded the study of the regulation of ureide metabolism at the molecular level. Interestingly, when the ureide concentration was determined in tissues from nodulated N\textsubscript{2}-fixing plants, only the roots showed a pattern of ureide accumulation resembling the nitrogen fixation kinetics, and neither gene expression nor ureide metabolism enzymatic activities changed significantly during plant development in roots, suggesting that they make a limited contribution to the generation of ureides upon the remobilization occurring after flowering.

In the present study, allantoate was the most abundant ureide in most tissues and under most conditions, except for roots that contained similar allantoin and allantoate levels. Except for roots, similar or even higher allantoin than allantoate levels were found in samples during early vegetative growth (Fig. 1). Remarkably, the presence of allantoin coincided with low ALN activity (Fig. 4). Induction of ALN activity, mainly in shoots, led to the reduction of allantoin and to the steady accumulation of allantoate, thus suggesting that AAH is limiting the rate of ureide degradation. This was previously suggested by Thomas and Schrader (1981), although neither gene expression analysis nor the assay of enzymatic activity able to degrade allantoate was feasible at that time. The results in Figs 3 and 4 strongly suggest that the induction of expression and activity of ALN, mostly in the stem, is responsible for
the significant increase in allantoate. It was noticeable that the ALN activity pattern did match the transcript levels. Similarly, low levels of AAH activity match the lower induction of *PvAAH* transcript expression, supporting the transcriptional regulation of ureide metabolism, although other levels of regulation could also be possible.

Despite the large number of reports studying ALN activity in plants, mainly in legumes, analysis of ALN gene expression is very limited. The results in Fig. 3A show that ALN expression is highly induced upon development, mainly in stems. Maximum ALN expression levels were reported in bark/cambial zone from black locust (*Robinia pseudoacacia*) shoots (Yang and Han, 2004), suggesting that preferential expression in shoots is also a feature for ALN in this non-ureide-type legume. The nitrogen source has been shown to regulate ALN expression in *Arabidopsis* (Werner et al., 2008), which apparently contradicts the present results showing small differences in transcript abundance in tissues from N2-fixing and nitrate-fed plants (Fig. 3). In *Arabidopsis*, the lowest mRNA level was found in nitrate-treated plants, and it increased in response to nitrogen starvation or allantoin feeding. However, it is unclear whether these results correspond to repression by nitrate or to induction by nitrogen starvation in the other conditions.

Whereas ALN activity agrees well with its transcript abundance in all tissues examined, the highest relative *AAH* gene expression was found in leaves, although they showed the lowest AAH activity, thus suggesting that some post-translational regulation of AAH activity could operate in this tissue. Post-translational regulation of AAH has already been suggested to explain the accumulation of allantoate observed under drought conditions when *AAH* gene expression was either maintained at constant levels (Charlson et al., 2009) or even induced (Alamillo et al., 2010).

In contrast to the changes in *PvAAH* presented here, the expression of *AAH* in *A. thaliana* was similar in all tissues and growth stages examined (Todd and Polacco, 2006), and upon N starvation or nitrate feeding (Werner et al., 2008). Whereas these studies point to constitutive *AAH* mRNA expression, the present results show that *PvAAH* mRNA expression is not affected by the nitrogen source, but it is regulated by the plant developmental stage. These discrepancies might be explained by the high sensitivity of the qRT-PCR used in this study, whereas the semi-quantitative RT-PCR used in previous reports may not be accurate enough to detect the moderate changes in *AAH* expression shown here.

Low allantoate-degrading capacity has been considered as the bottleneck for ureide metabolism (Thomas and Schrader, 1981), and the reason for the accumulation of ureides under drought stress in soybean (King and Purcell, 2005). However, data in common bean show that accumulation of ureides is, indeed, caused by the induction of allantoate synthesis through the positive regulation of ALN (Alamillo et al., 2010; this study). These results should be considered for future strategies aiming to improve nitrogen fixation (i.e. limiting ureide accumulation under drought stress) and suggest that they should focus on the control of ALN regulation, instead of looking for improved allantoate degradation.

The distribution of the ureide, amino acid, and nitrate content in xylem sap of *P. vulgaris* summarized in Table 2 and depicted in Fig. 6 strongly supported the generalized idea that ureidic plants behave as amodic plants upon nitrate fertilization (Pate et al., 1980). Furthermore, the concentrations of amino acids shown in Table 2 are similar to previously reported levels in nitrate-fertilized *Phaseolus* plants (Leidi and Rodriguez-Navarro, 2000). Nevertheless, although nitrate-fed plants transported more asparagine and glutamine in their xylem than plants fixing nitrogen, the significant levels of asparagine and glutamine measured during the pod-filling stage in the nodulated plants is still remarkable. Remobilization of nutrients in the oldest tissues is the most likely source of both amides and ureides found at late developmental stages.

Nitrogen remobilization has been reported to be preferentially partitioned to reproductive organs in pea and other grain legumes (Salon et al., 2001; Schiltz et al., 2005), although enhancement of nitrogen and CO2 fixation in pea nodules was also suggested as a mechanism to supply the high demands of early fruit development (Fischinger and Schulze, 2010). However, the present data show that nitrogen fixation in *Phaseolus* peaks several days before flowering, and, therefore, mobilization of N stored in vegetative tissues should satisfy the pod’s development demands. The results suggest that nitrogen mobilized from senescent tissues is mainly channelled through synthesis of ureides, thus supporting the idea of ureide-N having a predominant role in pod development. Ishizuka (1977) suggested that ureide-N, arising predominantly from N fixation, was used more efficiently in seed protein production than N in the form of amino acids, amides, and nitrate. According to this, ureides reached their highest levels in the developing fruits from *Phaseolus* (Raso et al., 2007b), also supporting a role for ureides in pod development.

In summary, results in this report show that the developmental stage influences ureide levels in *P. vulgaris*, both under nitrogen-fixing conditions and in nitrate-fed plants, and that changes in ureide levels during *P. vulgaris* development are mediated by the tissue-specific regulation of ALN. In addition, they suggest that remobilized N from lower leaves is involved in the sharp rise in ureides in shoots and leaves during early pod filling in N2-fixing common bean, and in the moderate, although significant, amounts of ureides observed in non-nodulated plants.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Purine catabolism in plants.

**Figure S2.** Specificity of primers used to determine the expression of *PvALN* genes.

**Figure S3.** Analysis of relative transcript expression of allantoinase genes during development of *Phaseolus vulgaris* plants grown under nitrogen fixation or nitrate-fertilized conditions.
Figure S4. Relative transcript expression of ureide synthesis and degradation genes during development in nodules from *P. vulgaris* plants.

Figure S5. Amino acid composition in the xylem sap from nodulated, N₂-fixing, and nitrate-fertilized *P. vulgaris* plants.

Table S1. Primers used for quantification of mRNAs from French bean tissues by qRT-PCR.

Table S2. Evolution of the N₂ fixation activity during the development of common bean plants.

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