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Research

Asparagine accumulation in chicory storage roots is controlled by translocation and feedback regulation of asparagine biosynthesis in leaves

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Summary

- The presence of acrylamide (AA), a potentially carcinogenic and neurotoxic compound, in food has become a major concern for public health. AA in plant-derived food mainly arises from the reaction of the amino acid asparagine (Asn) and reducing sugars during processing of foodstuffs at high temperature.
- Using a selection of genotypes from the chicory (*Cichorium intybus* L.) germplasm, we performed Asn measurements in storage roots and leaves to identify genotypes contrasting for Asn accumulation. We combined molecular analysis and grafting experiments to show that leaf to root translocation controls Asn biosynthesis and accumulation in chicory storage roots.
- We could demonstrate that Asn accumulation in storage roots depends on Asn biosynthesis and transport from the leaf, and that a negative feedback loop by Asn on *CiASN1* expression impacts Asn biosynthesis in leaves.
- Our results provide a new model for Asn biosynthesis in root crop species and highlight the importance of characterizing and manipulating Asn transport to reduce AA content in processed plant-based foodstuffs.

Introduction

Biosynthesis and accumulation of asparagine (Asn) in crop species have gained attention since the discovery that Asn is a precursor in the formation of acrylamide (AA) during processing of raw materials at temperatures above 120°C. In the liver, AA is metabolized to glycidamide, an epoxide that is more reactive to DNA and proteins than AA itself (Pedreschi *et al.*, 2014). Although AA is only classified as probably carcinogenic to humans (Group 2A), a recent study identified a mutational signature in human tumour tissues that appears imprinted by AA, through the effects of glycidamide (Zhivagui *et al.*, 2019). AA is formed through the Maillard reaction during the process of baking, roasting, frying, toasting, and microwaving (Mottram *et al.*, 2002; Stadler *et al.*, 2002). One of the major concerns about the presence of AA in foods is its presence in many foodstuffs consumed on a daily basis, such as French fries, potato crisps, bread, fine bakery wares, coffee, coffee substitutes, and baby food (Commission Regulation (EU) N2017/2158). In an attempt to prevent and reduce formation of AA during the industrial processing of foodstuffs, an acrylamide toolbox was created by FoodDrinkEurope (2019). This toolbox is based on four parameters: (1) agronomy, (2) recipe, (3) processing, and (4) final preparation. Because the strategies 2 and 3 often alter the properties (i.e. flavour, appearance, quality) of the processed product and are food system specific, exploitation of the natural variation in AA precursors present in plants appears as a promising route. Therefore, investigation of biosynthesis and accumulation of AA precursors in plant tissues/organs submitted to processing has become key to help reducing exposure to AA. The identification of genetic factors involved in the production, transport, and accumulation of AA precursors is particularly relevant to address this issue globally with improved varieties with low potential for AA production.

Asn is the most important determinant for AA formation as its concentration in raw materials has been shown to be directly correlated with AA formation in coffee (Bagdonaite *et al.*, 2008), wheat (Muttucumaru *et al.*, 2008), rye (Curtis *et al.*, 2010), and chicory (Loaëc *et al.*, 2014). Asn is also an important component for AA formation in potato, though reducing sugars appear to be the major factor (Zyzak *et al.*, 2003; Muttucumaru *et al.*, 2017). Strategies based on agricultural practices, natural variation and biotechnology have recently been investigated to reduce the AA potential of crop species (Loaëc *et al.*, 2014; Zhu *et al.*, 2016; Curtis *et al.*, 2018). Asn, besides being a proteinogenic amino acid, plays a central role in nitrogen metabolism in plants, acting

in nitrogen transport, recycling and remobilization during development (Gaufichon *et al.*, 2010). The main Asn biosynthetic pathway in plants involves the assimilation of ammonium into Asn through a series of reactions with the participation of four enzymes: glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AspAT), and Asn synthetase (ASN), all belonging to multigene families (Gaufichon *et al.*, 2010, 2015). Because ASN catalyses the final step of Asn biosynthesis, genetic engineering approaches to produce crop plants with low Asn content have relied on the organ-specific alteration of *ASN* expression (Rommens *et al.*, 2008; Chawla *et al.*, 2012). However, alteration of Asn biosynthesis can also lead to phenotypic variation and yield penalty (Rommens *et al.*, 2008). This reflects the importance of Asn homeostasis for the plant metabolism. It has also been demonstrated that overexpression of the protein kinase general control nonderepressible (GCN)2, a protein that phosphorylates the α subunit of eukaryotic translation initiation factor 2 could lead to a significant decrease of total free amino acids in wheat grains and a concomitant reduction in *ASN1* expression (Byrne *et al.*, 2012).

Although Asn is associated with nitrogen (N) remobilization *in planta* through transport in vascular tissues (Krapp, 2015; Tegeder & Hammes, 2018), the contribution of long-distance transport to Asn accumulation in crop plants has so far received limited attention. Chicory (*Cichorium intybus* L.) belongs to the Asteraceae family and is used as a root crop for the production of inulins and as a coffee substitute. The latter requires processing at high temperature and is prone to AA production. Previous studies have demonstrated that the AA content in processed chicory roots is directly correlated with the Asn concentration (Loaëc *et al.*, 2014). Recent advances in genome sequencing and the availability of genotypes from breeding programmes aiming at reducing their AA potential make chicory a species of interest to investigate Asn biosynthesis and transport. The production of storage root during chicory development also offers the opportunity to investigate the role of sink organs in the Asn metabolism.

Here, we investigated accumulation and transport of free Asn between chicory organs and their impact on Asn biosynthesis. For this purpose, we identified the Asn biosynthetic genes in the chicory genome and characterized genotypes for their Asn biosynthesis and accumulation in leaves and roots during plant development. We took advantage of grafting experiments and Asn measurement in genotypes contrasting for Asn biosynthesis and accumulation to identify a negative feedback regulation by Asn. We propose a model accounting for Asn biosynthesis and transport between organs. Our study highlights the importance of studying Asn transport in crop plants with high AA potential and opens new perspectives to modify Asn accumulation without altering its biosynthesis.

Materials and Methods

Contrasting genotypes for asparagine content in chicory (*Cichorium intybus* L.) germplasm

For this purpose, a field trial (year 1) was conducted at Cosucra Group Warcoing SA (Pottes, Belgium) from May to November of 2017 with 18 genotypes of *C. intybus* including S_3 to S_8 lines (L3427, L4108, L4115, L4118, L8014, L5040), inbred lines (inb14, inb40, inb42, VL54, VL57, VL59), hybrids (HYB50, OBOE), synthetic varieties (Larigot, Cadence, Hera) and a variety with uncharacterized genetic structure (Malachite). Storage roots of each genotype were harvested at 30, 60, 90, 120, 150 and 180 d after sowing (das) for analysis of the Asn content. The experimental design consisted of five randomized blocks where four to eight storage roots, in each block, were harvested and pooled for the analysis. The year 2 experiment was conducted with the genotypes L4115, L4118, inb42, L8014, HYB50, OBOE and Larigot that were harvested at 60, 120 and 180 das. For the second experiment (year 2), the experimental design consisted of five randomized blocks where six storage roots, in each block, were harvested and pooled for analysis.

Asparagine measurement

The determination of free Asn content in storage roots was carried out with an L-ASN kit (K-ASNAM; Megazyme, Bray, Ireland). Measurements were carried out according to Lecart *et al.* (2018) with some modifications. Briefly, 200 mg of freeze-dried and ground roots were mixed with 1.6 ml of 1 M perchloric acid and homogenized in a TissueLyser II (Qiagen) in microtubes containing six glass beads of 2.8 mm in diameter. Samples were centrifuged at 10 000 ***g*** for 20 min and 1 ml of the supernatant was collected and neutralized with 1 M potassium hydroxide until reaching pH 8.0 (*c.* 500 µl). The material was then placed on ice for 20 min before centrifugation at 10 000 ***g*** for 10 min. The supernatant was immediately used for Asn measurement according to the manufacturer's instructions for the microplate format. Each sample was quantified in triplicate.

Identification of asparagine biosynthetic genes

To identify the genes involved in the Asn biosynthetic pathway in chicory we took the advantage of restricted access to the chicory genome and RNA sequencing library from leaves and storage roots as well as a chicory expressed sequence tag (EST) database (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=13427). A BLAST approach was performed using orthologous sequences of ASN, GS, GOGAT, and AspAT retrieved from Arabidopsis (https://www.arabidopsis.org/), as well as Asteraceae family species (*Lactuca sativa* and *Helianthus annuus*) and Asterid clade species (*Solanum tuberosum* and *Solanum lycopersicum*) retrieved from the National Center for Biotechnology Information. All chicory sequences are deposited in GenBank: ASN 1 (*CiASN1*), MT470427; ASN 2 (*CiASN2*), MT470428, MT 470429, MT470430; cytosolic GS (*CiGLN1;1*), MT470431; cytosolic GS (*CiGLN1;2*), MT470432, MT476219; cytosolic GS (*CiGLN1;3*), MT470433; chloroplastic GS (*CiGLN2*), MT 470434; ferredoxin-dependent GOGAT (*CiGLU*), MT470435; NADH-dependent GOGAT (*CiGLT*), MT470436; AspAT, chloroplastic (*CiAspAT3*), MT476215; AspAT, mitochondrial (*CiAspAT1*), MT476216; AspAT, cytosolic (*CiAspAT2;1*), MT476217; AspAT, cytosolic (*CiAspAT2;2*), MT476218.

RNA extraction and quantitative reverse transcription PCR analysis

Total RNA was purified from freeze-dried ground leaves and roots. Up to 50 mg of plant material was lysed with buffer RLT (Qiagen, Antwerp, Belgium) followed by protein removal with chloroform : isoamyl alcohol (24 : 1) and RNA precipitation with isopropanol. The resulting RNA was washed with 70% ethanol and resuspended in RNase-free water. Quantification was performed using the Quantus Fluorometer (Promega, Leiden, the Netherlands), and RNA quality was checked by agarose gel electrophoresis. Subsequently, 2 µg of RNA for each sample was treated with DNase I at 37°C for 10 min (New England Biolabs, Leiden, the Netherlands), and 0.5 µg was used for reverse transcription with the GoScript Reverse Transcription Mix, Oligo dT (Promega, Leiden, the Netherlands). The concentrations of the resulting complementary DNAs were adjusted to 10 ng μl^{-1} and 2 µl was used for quantitative reverse transcription PCR (RT-qPCR) reactions performed with the GoTaq qPCR Master Mix (Promega) in a final volume of 10 µl. Amplification was detected using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Temse, Belgium). To further identify stable reference genes to our experimental conditions (namely, storage roots, leaves, and contrasting genotypes to Asn), a normalization experiment was conducted with reference genes available in the literature for chicory. Primer sequences for actin (*ACT*), elongation factor 1-alpha (*EF*), histone H3 (*H3*), and 18S rRNA (*rRNA*) were retrieved from Maroufi *et al.* (2010), whereas protein phosphatase 2A subunit A2 (*PP2AA2*), protein phosphatase 2A subunit A3 (*PP2AA3*), SAND family protein (*SAND*), TIP41-like protein (*TIP41*), and ubiquitin-conjugating enzyme (*UBC*) were retrieved from Delporte *et al.* (2015). A score was attributed to each reference gene according to their stability in geNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), and BestKeeper (Pfaffl *et al.*, 2004) software and a ranking from the most stable genes (lowest score) to the most unstable (highest score) was performed (Supporting Information Fig. S1). The optimal number of reference genes for storage roots and leaves was chosen according to the pairwise variation V from geNorm (Fig. S2), and the selected genes were chosen based on the highest stability according to the ranking (Fig. S1). Primer sequences for all the chicory genes used in this study are provided in Table S1.

Grafting

The experiment was conducted under glasshouse conditions. A protocol for chicory grafting was established as depicted in Fig. S3. Seeds were germinated in the dark for 5 d to promote stem growth. The seedlings were subsequently transferred to a 16 h : 8 h, light : dark, photoperiod for 1 month before grafting. Cotyledons and leaves were removed and plantlets used for grafting by the cleft method. The cuttings for the grafting were performed with a razor blade under a binocular stereoscopic microscope. The junctions were covered with a porous tape (Micropore™; 3M, Neuss, Germany) and placed high above the substrate in 3-l pots. A humid environment was created by covering the pots with transparent bags that were removed after 14 d. Ammonium nitrate (100 mg) was added to each pot 1 month after grafting. Plants were harvested after 3 months; leaves and storage roots were collected, immediately frozen in liquid N_2, and then freeze-dried for use in biochemical and molecular analyses. Analyses were performed on three to eight biological replicates for each grafting combination.

Leaf infiltration with amino acids

In vitro chicory plantlets at 45 d old, grown at 25°C under a photoperiod of 16 h : 8 h, light : dark, were used for leaf infiltration of amino acids. Asn (190 mM), aspartate (Asp; 37 mM), glutamate (Glu; 57 mM), and glutamine (Gln; 190 mM), individually solubilized in water, were infiltrated with a 6 ml syringe (Norm-ject™, Tuttlingen, Germany) into chicory leaves until they were completely saturated with the respective solution. These concentrations were chosen according to the maximum solubility in water of each amino acid. After injection, plants were kept in the growth chamber for 36 h. After this period, leaves were harvested to proceed with gene expression analysis. Each treatment was conducted in four biological replicates.

Protoplasts feeding with asparagine

Protoplasts from leaves were isolated according to Deryckere *et al.* (2012). The protoplasts were resuspended in washing and incubation solution – 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM potassium chloride – and a total of 34 µl containing 8×10^6 cells were placed in 24-well plates. Then, 216 µl of a 220 mM Asn solution was added to perform a final concentration of 190 mM in a final volume of 250 µl for protoplast feeding assay. The protoplast plates were incubated at room temperature for 36 h. Protoplasts were subsequently used for RNA extraction and gene expression analysis.

Results

Diversity of asparagine content in chicory germplasm

We first identified genotypes contrasting for Asn content in storage roots to compare the specificities of lowAsn (LAsn) and high-Asn (HAsn) chicory genotypes at the molecular level. The germplasm bank included lines, hybrids, and synthetic varieties. Our analysis revealed genotypes with Asn content in storage roots ranging from 27.3 ± 5.2 mg per 100 g (L4115) to 209.8 ± 35.8 mg per 100 g (L4118) DW 150 das before the initiation of senescence (Fig. 1a). A classification was also performed with the Asn content at 180 das (Fig. S4). Based on the Asn content at 150 das, the genotypes could be divided into four groups according to their Asn levels in storage roots: LAsn content, medium Asn (MAsn) content, medium-high Asn (MHAsn) content, and HAsn content. The Asn content in storage roots did not correlate with root diameter ($r = -0.03$) and root weight ($r = -0.05$) (Fig. S5), indicating that the Asn levels could not be explained by the phenotypic variation at harvest (Fig. S6).

Noticeably, the genotype classified as LAsn displayed stable Asn levels from 30 das until harvest (Fig. S7). On the contrary, Asn levels increased over time in genotypes classified as MAsn, MHAsn, and HAsn (Fig. S7). Several genotypes accumulated increasing levels of Asn in storage roots until harvest, whereas Asn levels appeared to peak and reach a plateau before harvest in other genotypes (Fig. S7). A second-year field experiment was conducted to confirm Asn accumulation profiles from selected genotypes. The selected genotypes displayed similar trends in Asn accumulation despite lower levels observed in the second-year field trial (Fig. S8), possibly due to environmental variation as previously reported (Loaëc *et al.*, 2014). In particular, differences in N availability could have occurred due to reduced precipitation and drier conditions during the second year.

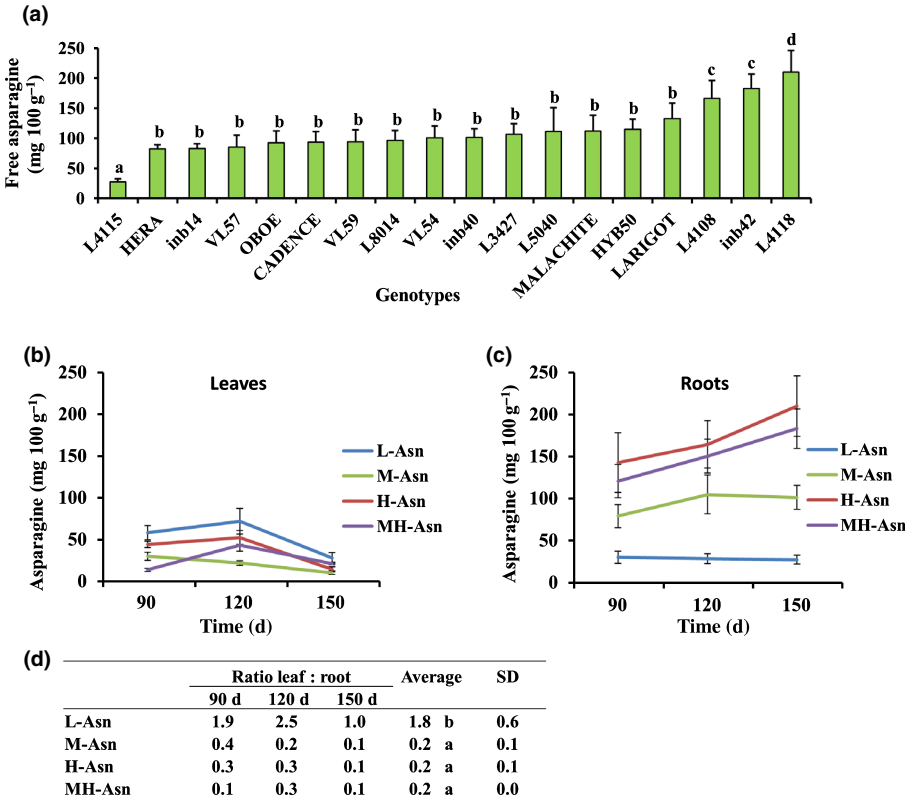
We next measured the Asn content in leaves from the genotypes contrasting for Asn accumulation in storage roots (L4115, inb40, inb42 and L4118, referred to as LAsn, MAsn, MHAsn and HAsn, respectively, from now) to investigate the relation between Asn contents in storage roots and leaves (Fig. 1b,c). By computing the $[Asn]_{leaf}/[Asn]_{storage\ root}$ ratio, we found that the genotype LAsn had a significantly higher ratio (1.8) than the other genotypes (0.2; Fig. 1d). Moreover, we found a significant negative correlation ($r = -0.41$, $P = 0.007$) between $[Asn]_{leaf}$ and $[Asn]_{storage\ root}$ in those genotypes (Fig. S9).

Asparagine biosynthetic pathway in chicory

Having identified chicory genotypes displaying a variation in both $[Asn]_{leaf}$ and $[Asn]_{storage\ root}$, we next identified Asn biosynthetic genes in the chicory genome. Chicory genomic sequences were BLAST searched using Asn biosynthetic genes from Arabidopsis in order to identify gene members of each family involved in the biosynthesis of Asn in chicory. We identified two genes coding for ASN (*CiASN1* and *CiASN2*), four genes coding for GS (*CiGLN1;1*, *CiGLN1;2*, *CiGLN1;3* and *CiGLN2*), two genes coding for GOGAT (*CiGLU1* and *CiGLT*) and four genes coding for AspAT (*CiAspAT1*, *CiAspAT2;1*, *CiAspAT2;2* and *CiAspAT3*) (Fig. 2a). The gene families from Asn biosynthetic pathway appeared to be relatively conserved in size, the differences between species mostly reflecting the differences in ploidy levels (Fig. S10; Van Bel *et al.*, 2018). However, it should be noted that our identification of Asn biosynthetic genes might have been partial because it relied on data from preliminary assemblies of chicory genomic sequences obtained through a shotgun sequencing approach (unpublished data) and sequences from publicly available chicory ESTs. The *in silico* molecular analysis identified Asn biosynthetic genes with expression in both storage roots and leaves from selected chicory genotypes. In storage roots, the gene members coding for ASN, GS, GOGAT and AspAT that displayed the highest expression were *CiASN2, CiGLN1;3, CiGLT* and *CiAspAT2;2*, respectively (Fig. 2b). Four gene members (*CiASN2, CiGLN2, CiGLU* and *CiAspAT3*) did not present differential expression in storage roots in the contrasting genotypes, whereas others (*CiGLN1;3*, *CiGLT* and *CiAspAt2;2*) were downregulated in the genotype HAsn. Overall, Asn accumulation in storage roots from contrasting chicory genotypes did not correlate with transcripts levels of Asn biosynthetic genes in their storage roots. On the contrary, seven gene members (*CiASN1, CiGLN1;1, CiGLN1;2, CiGLN2, CiGLU, CiAspAT2;1* and *CiAspAT3*), representatives of all the Asn biosynthetic families, displayed expression levels that were significantly higher in the leaves of the HAsn genotype than in the leaves of the LAsn genotype (Fig. 2c). These results suggest that the high levels of Asn accumulation in storage roots could result from Asn synthesis in leaves and Asn transport to the storage roots. Noticeably, leaves of the HAsn genotype accumulated lower levels of Asn than the leaves of the LAsn genotype did, suggesting differential source–sink transport of Asn in those genotypes.

Synthesis of asparagine in leaves highly influences the pool of asparagine in roots

To further explore Asn transport and accumulation in source and sink organs, we took advantage of the contrasting genotypes to determine the contribution of leaf Asn biosynthesis to the Asn pool in storage roots. We established a protocol enabling successful chicory grafting (Fig. S3), and we performed heterografting of LAsn and HAsn genotypes (Fig. S11). Three months after grafting, we measured $[Asn]_{leaf}$ and $[Asn]_{storage\ root}$ in the reciprocal heterografts LAsn/HAsn and HAsn/LAsn as well as in the control homografts (Fig. S11). The HAsn homografts accumulated Asn levels in storage roots that were over two times higher than in the LAsn homografts, indicating that the newly established grafting model is suitable to measure differences in Asn accumulation and transport between organs (Fig. 3a). The $[Asn]_{storage\ root}$ from the heterografts LAsn/HAsn was as low as in the homografts LAsn (Fig. 3a), confirming the contribution of leaf Asn biosynthesis to Asn accumulation in HAsn storage roots genotypes. Intriguingly, the HAsn/LAsn heterografts did not lead to high $[Asn]_{storage\ root}$ (Fig. 3a). In addition, $[Asn]_{leaf}$ in the HAsn/LAsn heterografts was also reduced compared with $[Asn]_{leaf}$ in the HAsn homografts (Fig. 3b). On the contrary, $[Asn]_{leaf}$ in the LAsn/HAsn heterografts remained unaltered compared with the LAsn homografts. We subsequently measured and compared expression levels of *CiASN1* and *CiASN2* genes in leaves from both the HAsn homografts and the HAsn/LAsn heterografts. *CiASN1* displayed a significantly higher expression in HAsn homografts leaves than in HAsn/LAsn heterografts, but *CiASN2* remained unaltered (Fig. 3c). This observation suggests that the sink organ impacts expression of Asn biosynthetic genes in the source organ. The low Asn levels in the LAsn storage roots from the HAsn/LAsn heterografts also suggest that LAsn storage roots are impeded in transport and/or accumulation of Asn. Because leaves in the HAsn/LAsn heterografts accumulate intermediary Asn amounts (Fig. 3b), we hypothesized that a limited transport and accumulation of Asn in storage roots could increase Asn levels in leaves, and thus negatively impact the Asn biosynthesis there. To test this hypothesis, we established an *in vivo* experiment in which the Asn, Asp, Gln, and Glu amino acids were individually infiltrated in chicory leaves. Thirty-six hours after the infiltration, RNA was isolated from the infiltrated leaves and the expression



(d)

	Ratio leaf : root			Average		SD
	90 d	120 d	150 d			
L-Asn	1.9	2.5	1.0	1.8	b	0.6
M-Asn	0.4	0.2	0.1	0.2	a	0.1
H-Asn	0.3	0.3	0.1	0.2	a	0.1
MH-Asn	0.1	0.3	0.1	0.2	a	0.0

Fig. 1 Diversity of asparagine (Asn) accumulation in the *Cichorium intybus* germplasm. (a) Free Asn content (milligrams per 100 g DM) in storage roots of selected chicory genotypes 150 d after sowing. Data are mean ± SD of five biological replicates, each one representing an average of four or eight storage roots. Statistically significant differences are represented by different letters (one-way ANOVA, $P < 0.05$ followed by Scott–Knott test, $P < 0.05$). Genotypes with the letters a, b, c and d were classified as low Asn (LAsn) content, medium Asn (MAsn) content, medium-high Asn (MHAsn) content, and high Asn (HAsn) content, respectively. (b, c) Free Asn accumulation (milligrams per 100 g DM) in (b) leaves and (c) storage roots from selected contrasting genotypes at 90, 120 and 150 d after sowing. Each point represents the mean ± SD of five biological replicates. For more details of other genotypes, see Supporting Information Fig. S7. (d) Ratio of free Asn content between leaves and storage roots of selected contrasting genotypes at 90, 120 and 150 d after sowing.

levels of gene members were evaluated by RT-qPCR. As a control, we analysed leaves infiltrated with water. The infiltration of Asn into chicory leaves led to the downregulation of *CiASN1*, *CiGLN1;1* and *CiGLU* but had no effect on *CiASN2*, *CiAspAT2;1* and *CiAspAT3* (Fig. 3d). We confirmed the downregulation of *CiASN1* by Asn feedback inhibition using a protoplast assay (Fig. S12). On the contrary, leaf infiltrations of Gln, Glu, and Asp led to significant increased expression of *CiASN1*, whereas expression of *CiASN2* remained unchanged compared with controls (Fig. 3e–g). These results differ from *AtASN1* and *AtASN2* expression profiles in Arabidopsis leaves as N metabolites, including Asn, Gln and Glu, upregulate *AtASN1* and downregulate *AtASN2* (Lam *et al.*, 1998). However, sequence analysis suggests that *CiASN1* and *CiASN2* are the functional orthologues of *AtASN1* and *AtASN2*, respectively (Notes S1). To further characterize the Asn model in chicory, we quantitated Gln in the grafted plants. Gln serves as substrates for Asn biosynthesis, and we found that $[Gln]_{storage\ root}$ in the LAsn/HAsn heterografts was significantly reduced compared with the HAsn homograft (Fig. 3a). This suggests that the Gln pool in chicory storage roots is also influenced by the Gln biosynthesis and transport from the leaves. In addition, the $[Gln]_{leaf}$ in the HAsn/LAsn heterograft was significantly reduced compared with the HAsn homograft (Fig. 3b). Because the Asn infiltration in leaves also led to the downregulation of *CiGLN1;1* (Fig. 3b), our results suggest that the decrease in Asn biosynthesis and accumulation could be a consequence of the deregulation of several Asn biosynthetic genes in chicory leaves. The mounting concentration of Asn in chicory leaves due to limited transport and accumulation in the storage roots from the HAsn/LAsn heterografts could initiate and exert a negative feedback loop regulation on expression of Asn biosynthetic genes in HAsn leaves.

Discussion

Asn is considered to serve as a major transport compound in the xylem from the root to the leaves and in the phloem from the leaves to the developing seeds in several model and leguminous species that have been investigated (Amarante *et al.*, 2006; Lea *et al.*, 2007; Krapp, 2015). This model is also corroborated by the high N to carbon (C ratio of Asn (Coruzzi, 2003). Previous studies aiming at determining the AA potential in crop species have revealed that genetic variability exists in the accumulation of Asn in plant organs (Halford *et al.*, 2012; Postles *et al.*, 2013;

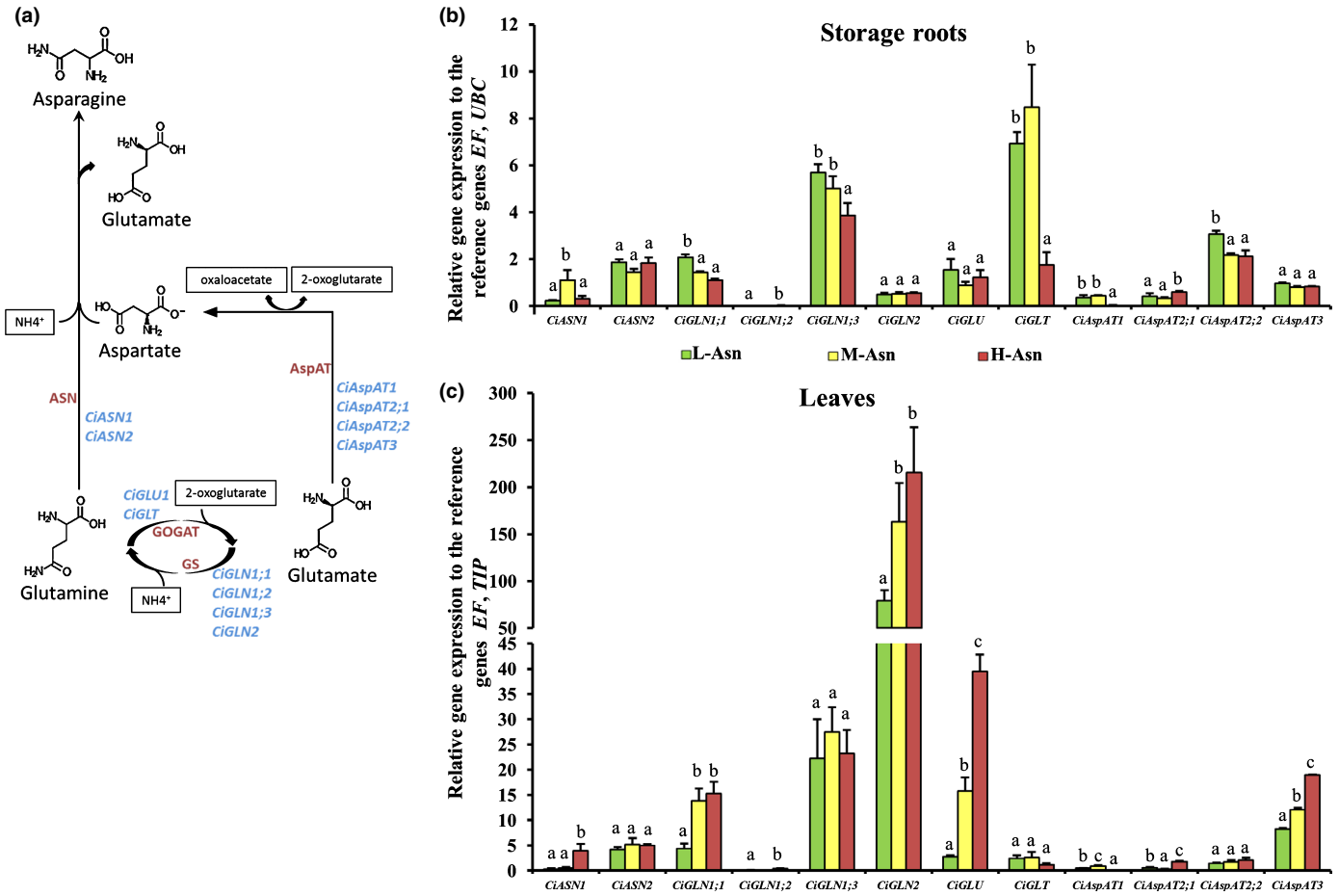


Fig. 2 Correlation between asparagine (Asn) content and the expression of the Asn biosynthetic genes in *Cichorium intybus*. (a) General overview of the Asn biosynthetic pathway in plants: Asn synthetase (ASN), glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AspAT); and the corresponding gene members identified in *C. intybus*: ASN (*CiASN*); cytosolic GS (*CiGLN1*); chloroplastic GS (*CiGLN2*); ferredoxin-dependent GOGAT (*CiGLU*); NADH-GOGAT (*CiGLT*); mitochondrial AspAT (*CiAspAT1*); cytosolic AspAT (*CiAspAT2*); chloroplastic AspAT (*CiAspAT3*). (b, c) Relative gene expression of Asn biosynthetic genes in (b) storage roots and (c) leaves of *C. intybus* from genotypes with low, medium and high Asn content (LAsn, MAsn and HAsn, respectively). *EF*, elongation factor 1-alpha; *UBC*, ubiquitin-conjugating enzyme. Data are mean ± SD of three or four biological replicates, each one representing an average of four plants. Different letters for each gene member represent statistical significance according to one-way ANOVA, $P < 0.05$ followed by Scott–Knott test, $P < 0.05$.

Curtis & Halford, 2016; Muttucumaru *et al.*, 2017; Curtis *et al.*, 2018). In the present study, we could identify chicory genotypes contrasting for Asn accumulation in storage roots, and with this support the hypothesis that diversity in Asn accumulation relates to the expression of Asn biosynthetic genes. Noticeably, we found a positive correlation between the gene expression of the Asn biosynthetic genes in leaves and the Asn level in storage roots (Fig. 2b,c), suggesting that Asn biosynthesis in leaves has an important contribution to the Asn pool in storage roots. This feature could be organ and species specific, as suggested by previous studies in potato demonstrating that tuber accumulation of Asn mainly occurs through Asn biosynthesis in tubers with minor contribution from the leaves (Chawla *et al.*, 2012). In addition, analysis of the $[Asn]_{leaf}/[Asn]_{storage\ roots}$ in the contrasting genotypes showed that the ratio is significantly higher for the LAsn genotype. Therefore, we suggest that the Asn balance between source and sink controls the Asn transport pathway and that for LAsn accumulators there is a limitation imposed by the excess of Asn in the resource supply. It can be hypothesized that the sink strength for Asn in chicory storage roots is variable across genotypes, the MAsn, MHAsn and HAsn genotypes harbouring the strongest root sinks. Such control on Asn transport operated by the sink has previously been reported in rice in which the spikelets appear to act as strong sinks for Asn that is sourced from the flag leaves (Yabuki *et al.*, 2017).

Our grafting experiment with genotypes contrasting for Asn accumulation demonstrates that a reduction in the biosynthesis of Asn in the leaves leads to a reduction in the Asn accumulation in the storage roots (heterografts LAsn/HAsn, Fig. 3a). Notably, in the referred experiment, Gln followed the same trend of accumulation in the leaves and storage roots. The expression profile of Asn biosynthetic genes in contrasting genotypes (Fig. 2b,c) further indicates that the pool of Asn in the storage roots originates mainly from the Asn synthesized and transported from the leaves. Moreover, our experimental data also show that the accumulation of Asn in storage roots plays a regulatory role on the

(a)

Asparagine and glutamine (mg per 100 g DW) in grafted STORAGE ROOTS

Treatments	Scion/Rootstock	Asn		Gln	
Grafting 1	L-Asn/L-Asn	136.9 ± 28.4	**a**	73.9 ± 9.8	**a**
Grafting 2	H-Asn/H-Asn	310.4 ± 42.3	**b**	424.9 ± 47.9	**c**
Grafting 3	L-Asn/H-Asn	148.7 ± 31.9	**a**	144.8 ± 25.7	**b**
Grafting 4	H-Asn/L-Asn	159.7 ± 45.2	**a**	94.6 ± 14.7	**a**

(b)

Asparagine and glutamine (mg per 100 g DW) in grafted LEAVES

Treatments	Scion/Rootstock	Asn		Gln	
Grafting 1	L-Asn/L-Asn	49.0 ± 14.9	**a**	92.3 ± 19.4	**a**
Grafting 2	H-Asn/H-Asn	479.3 ± 166	**c**	615.4 ± 11.9	**b**
Grafting 3	L-Asn/H-Asn	58.3 ± 9.6	**a**	113.7 ± 16	**a**
Grafting 4	H-Asn/L-Asn	106.6 ± 30.5	**b**	101.5 ± 18.9	**a**

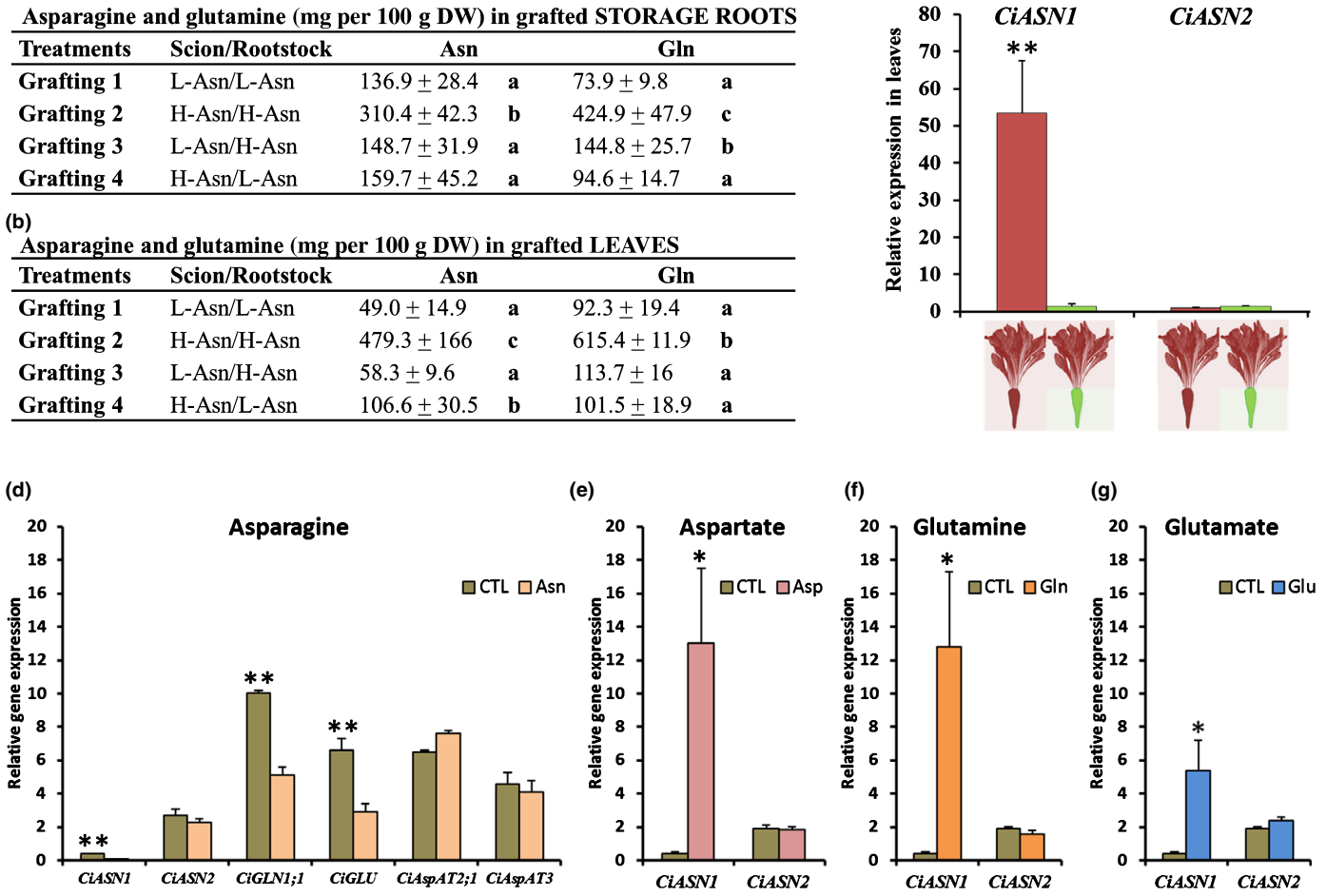


Fig. 3 Grafting of *Cichorium intybus* genotypes contrasting for asparagine (Asn) biosynthesis and accumulation. Free Asn content in (a) storage roots and (b) leaves of grafted plants at 3 months after grafting, expressed in milligrams per 100 g DW. A detailed scheme of the grafting experiment is depicted in Supporting Information Fig. S3. LAsn, low Asn; HAsn, high Asn. Data are mean ± SD representative of three to eight biological replicates, each one representing one plant. (c) Relative gene expression of the Asn synthetase (*ASN*) gene family in leaves from the graftings 2 (red/red) and 4 (red/green). (d–g) Relative gene expression of Asn biosynthetic genes in leaves infiltrated with (d) 190 mM Asn, (e) 37 mM aspartate (Asp), (f) 190 mM glutamine (Gln), and (g) 57 mM glutamate (Glu). Plants infiltrated with water were used as control. CTL, control; L-Asn, genotype L4115; H-Asn, genotype L-4118. Data are mean ± SD of four biological replicates, each one representing one plant. Statistical significance according to the *t*-test: *, $P < 0.05$; **, $P < 0.01$.

Asn biosynthetic pathway in the leaves (Fig. 3a,b). The observation that $[Asn]_{storage\ root}$ in the HAsn/LAsn heterograft was as low as in the homografts LAsn (Fig. 3a) suggests that the functionality of the Asn transport system in chicory is dependent on the activity of transporters in both leaves (for phloem loading) and storage roots (for phloem unloading through the apoplasmic pathway and/or post-phloem-transport via the apoplasm). Although the apoplasmic pathway of phloem unloading for C and N photoassimilates remains poorly understood, studies in crop and model species have demonstrated a key role of the apoplasm in the transport of hexose and amino acids (McCurdy *et al.*, 2010; Besnard *et al.*, 2016; Milne *et al.*, 2017). Transporters from the UMAMIT family have also been shown to be important in the post-phloem-transport of amino acids via the apoplasm (Müller *et al.*, 2015). We hypothesize that regulation of Asn transport in the phloem from loading to unloading steps contributes to the genetic diversity in Asn accumulation in chicory. Our grafting experiment also suggests that the accumulation of Asn in phloem tissues and/or the xylem transport of Asn from roots to leaves could contribute to a feedback regulation of the Asn biosynthesis in leaves. The reduction of Asn accumulation in the leaves of the heterograph HAsn/LAsn compared with HAsn homograft (Fig. 3b), was also concomitant with a significant reduction in the leaf expression of *ASN1* (Fig. 3c). One hypothesis that can be derived from our aforementioned observations is that the reduced phloem unloading of Asn in the rootstock of the HAsn/LAsn heterograft causes a negative feedback loop in the Asn biosynthetic genes in the leaves. Our leaf infiltration and protoplast experiments support a negative regulation of several Asn biosynthetic genes (i.e. *ASN1*, *GLN1;1* and *GLU*) by Asn in chicory (Fig. 3d). It is notable that the expression levels of the *ASN1*, *GLN1;1* and *GLU* genes followed the same trend in the leaves for the different genotypes studied (Fig. 2c), suggesting that they can act synergistically for the Asn synthesized in the leaves and transported to the storage roots. Pioneering work in Arabidopsis has demonstrated inducing and repressive action of sucrose on,

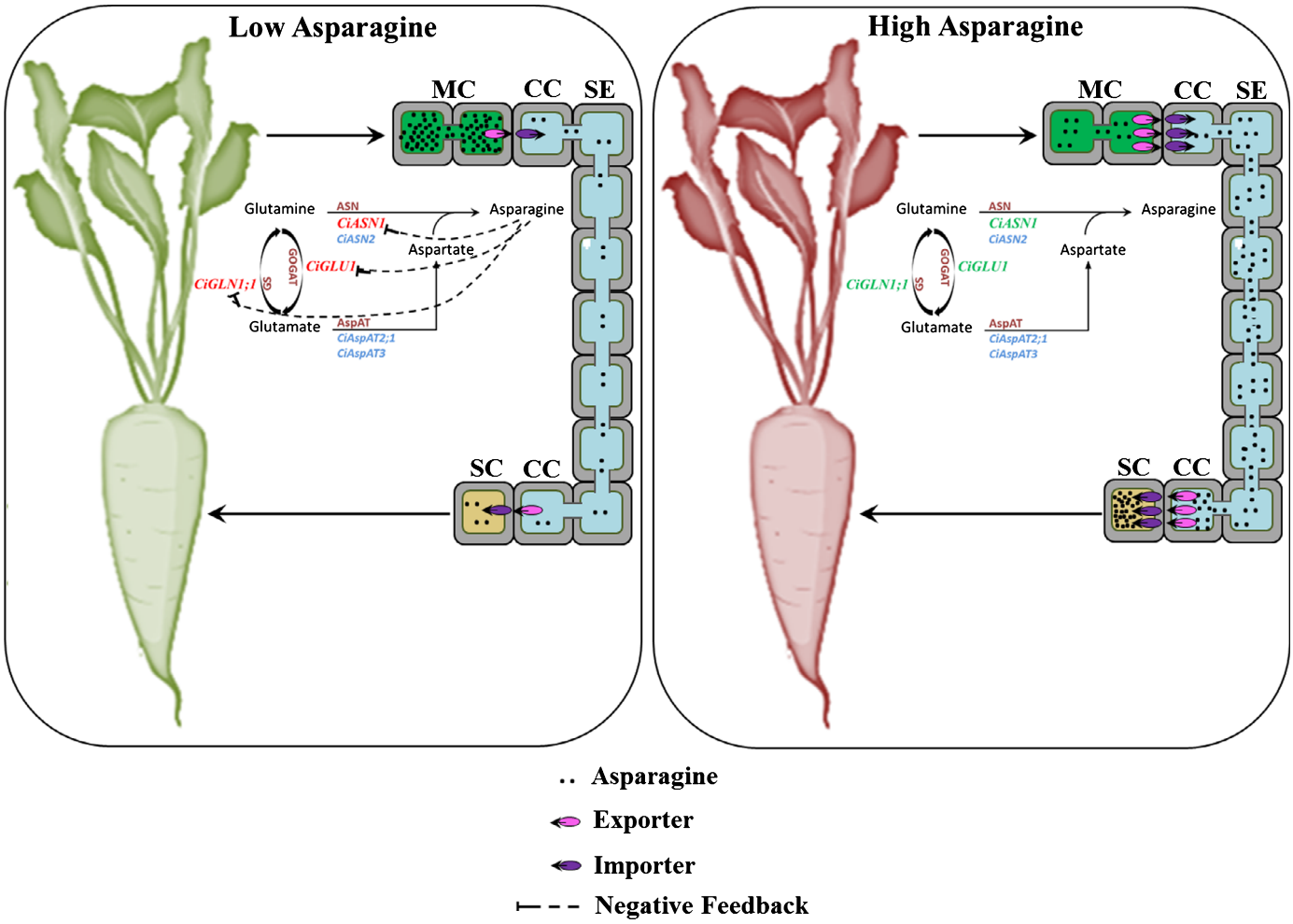


Fig. 4 Simplified model for asparagine (Asn) transport and accumulation in *Cichorium intybus* storage roots. In our model, plants with low Asn content (represented in green) either lack or have lower transporter (importers and exporters) activity controlling the Asn transport from leaves to storage roots. Thus, Asn accumulates in leaves and, in turn, downregulates *CiASN1* and other genes in the Asn biosynthetic pathway. The overall outcome of this is that less Asn will be transported to the storage roots. On the contrary, plants that accumulate high Asn levels in storage roots (represented in red) have a high activity of the transport system in leaves and storage roots, enhancing the transport of Asn from leaves to storage roots. The outcome is that *CiASN1* and other genes in the biosynthetic pathway can be expressed at high levels in leaves and thus produce more Asn that can be efficiently transported and stored. Our results showed that *CiASN2* is expressed at similar levels in the leaves of both (low and high-Asn) genotypes and is not controlled by the amino acids of the Asn biosynthetic pathway. Therefore, *CiASN2* is proposed to be involved mainly in the role of ammonium ion detoxification in chicory, as observed in potato (Chawla *et al*., 2012) and Arabidopsis (Wong *et al*., 2004). ASN, Asn synthetase; AspAT, aspartate aminotransferase; CC, company cells; GOGAT, glutamate synthase; MC, mesophyll cells; SC, storage cells; SE, sieve elements.

respectively, *ASN1* and *ASN2* transcript levels as well as the partial reversion of those alterations when supplementing Asn to seedlings (Lam *et al*., 1994, 1998). Though studies in other plant species have shown the regulatory potential of certain amino acids on their biosynthetic pathway (Lam *et al*., 1998; Gutiérrez *et al*., 2008; Zhang *et al*., 2015), further investigations are required to verify whether the Asn negative feedback regulation we characterized in chicory can be expanded to other plant species.

Based on the aforementioned results, we elaborated a simplified model depicting Asn biosynthesis and accumulation in chicory leaves and storage roots (Fig. 4). In this model, the pool of Asn accumulating in storage roots originates mainly from the pool of Asn produced and transported from the leaves. Thus, plants with a defective transport system, either in the leaves or both leaves and storage roots, accumulate high $[Asn]_{leaf}$, which in turn downregulates the expression of Asn biosynthetic genes. On the contrary, plants possessing an efficient transport system can translocate Asn into the storage roots, hence keeping $[Asn]_{leaf}$ low and thereby limiting the negative feedback loop on the expression of Asn biosynthetic genes.

Using chicory genotypes contrasting for Asn accumulation, we could demonstrate that Asn accumulation in storage roots depends on Asn biosynthesis in the leaf. Based on grafting experiments, leaf infiltration of amino acids, and gene expression studies, our results suggest that *CiASN1* is a key player for Asn biosynthesis in the leaf and that Asn acts as a repressor of *CiASN1* expression. Our model indicates that Asn transport and biosynthetic gene regulation by Asn are key mechanisms controlling Asn accumulation in plant organs. It should be mentioned

that other factors could play a role in the homeostasis and transport of Asn. Additional signal molecules, including hormones, should be further investigated, as the model is likely to be more complex. However, the feedback regulation between source and sink we report for the first time in chicory is likely to open new breeding perspectives to generate crop varieties with reduced AA potential. Our findings also highlight long-distance transport of amino acids and Asn transporters as central targets to reduce accumulation of free Asn in storage organs.

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Author contributions

Conceptualization: ES and HV; methodology: ES, LS and HV; validation: ES; formal analysis: ES; investigation, ES, LS, ND and CP; resources: HV, CN and OM; writing of original draft: ES and HV; writing – review and editing: ES, HV, LS, ND and CN; funding acquisition: HV, ES and OM; supervision: HV.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Selection of the best reference genes for normalization in *Cichorium intybus* storage roots and leaves.

Fig. S2 Determination of the optimal number of reference genes in *Cichorium intybus*, according to the pairwise variation V from geNorm.

Fig. S3 Grafting method for *Cichorium intybus*.

Fig. S4 Diversity of Asn accumulation in the *Cichorium intybus* germplasm.

Fig. S5 Correlation between diameter (cm) and Asn content (mg $100\ g^{-1}$) (a); weight (g) and Asn content (mg $100\ g^{-1}$) of storage roots in *Cichorium intybus*.

Fig. S6 Phenotypic variation of diameter and weight in *Cichorium intybus* storage roots 180 days after sowing.

Fig. S7 Free Asn content in storage roots from 18 *Cichorium intybus* genotypes measured at 30, 60, 90, 120, 150 and 180 days after sowing.

Fig. S8 Stability evaluation, over 2 years, of the Asn content in 7 genotypes of *Cichorium intybus* representative of the three contrasting groups to Asn level in storage roots.

Fig. S9 Correlation between Asn content (mg $100\ g^{-1}$) in leaves and storage roots of *Cichorium intybus*.

Fig. S10 Number of genes from the Asn biosynthetic pathway in *Arabidopsis thaliana* and crop plant species with high acrylamide potential.

Fig. S11 Overview of the grafting experiment with *Cichorium intybus*.

Fig. S12 Asn feeding experiment in *Cichorium intybus*.

Notes S1 Orthology analysis of *CiASN1* and *CiASN2*.

Table S1 Primer sequences used for the amplification of genes involved in the Asn biosynthetic pathway in *Cichorium intybus* and in the normalization experiment.

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