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REVIEW PAPER

# Regulation of amino acid metabolic enzymes and transporters in plants

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## Abstract

**Amino acids play several critical roles in plants, from providing the building blocks of proteins to being essential metabolites interacting with many branches of metabolism. They are also important molecules that shuttle organic nitrogen through the plant. Because of this central role in nitrogen metabolism, amino acid biosynthesis, degradation, and transport are tightly regulated to meet demand in response to nitrogen and carbon availability. While much is known about the feedback regulation of the branched biosynthesis pathways by the amino acids themselves, the regulation mechanisms at the transcriptional, post-transcriptional, and protein levels remain to be identified. This review focuses mainly on the current state of our understanding of the regulation of the enzymes and transporters at the transcript level. Current results describing the effect of transcription factors and protein modifications lead to a fragmental picture that hints at multiple, complex levels of regulation that control and coordinate transport and enzyme activities. It also appears that amino acid metabolism, amino acid transport, and stress signal integration can influence each other in a so-far unpredictable fashion.**

**Key words:** Amino acid, feedback regulation, membrane, metabolism, plant, regulation, transcriptional level, transport.

## Introduction

### *Importance and role of amino acids in the plant*

Amino acids are best known as constituents of proteins, and their central role in cellular and plant physiology is often overlooked. Pioneering experiments (Miller, 1953) and more recent research have suggested that amino acids could be among the first metabolites created by organisms (see Hernandez-Montes *et al.*, 2008), possibly explaining their involvement in most metabolic pathways and cellular processes. Amino acid metabolism is tightly linked to carbohydrate metabolism, ammonium (absorbed and synthesized from nitrate), and demand for protein synthesis and secondary metabolism. Amino acid biosynthesis uses compounds from carbohydrate metabolism, and amino acid degradation leads to several metabolites that are used by the citric acid cycle as an energy source. Furthermore, synthesis of the amino acid Gln is the only reaction allowing assimilation of inorganic nitrogen into organic molecules. All the pathways leading to the synthesis of all other nitrogenous compounds connect at some point with Gln or its sister metabolite, Glu (Bernard and Habash, 2009). As in animals and microbes, several amino acids play key roles in plants as precursor compounds for the synthesis of various classes of secondary metabolites (e.g. phenylpropanoids, some alkaloids, and glucosinolates). Secondary metabolites are extremely diverse (D'Auria and Gershenzon, 2005), and fulfil critical functions in the plant such as signalling (e.g. hormones), structure (e.g. lignin), defence (e.g. glucosinolates, nicotine), interaction with other organisms, and protection (e.g. pigments). Finally, amino acids are used as carriers of assimilated nitrogen between the various organs through both the phloem and the xylem. For instance, due to

Abbreviations: AAP, amino acid permeases; ABA, abscisic acid; ACT, amino acid/choline transporter; ANT, neutral amino acid transporter; APC, amino acid–polyamine–choline; bZIP, basic leucine zipper; CAT, cationic amino acid transporter; GABA, γ-aminobutyric acid; GAT, γ-aminobutyric acid transporter; GLR, glutamate-like receptor; LHT, lysine and histidine transporter; ProT, proline transporter.

the limited photosynthetic activity of the seed, amino acids used for synthesis of storage proteins are not synthesized *de novo* in the embryo. Amino acids are instead provided by the leaves and transported to the developing embryo through the xylem and phloem (Peoples *et al.*, 1985). At the whole-plant level, amino acids cycle between roots and shoots, transported alternatingly by the phloem and the xylem (Jeschke and Pate, 1991; Jeschke and Hartung, 2000). Changing concentrations of ascending or descending amino acids are thought to transduce nitrogen availability and demand from the shoots to the roots (Imsande and Touraine, 1994). Amino acids also play a crucial role during pathogen infection, being an indispensable source of nitrogen for many biotrophic pathogens (Douglas, 1993; Solomon *et al.*, 2003; Rico and Preston, 2008) and as providers of defence compounds. Not surprisingly, pathogen infection has been shown to lead to changes in expression of many genes involved in amino acid metabolism and transport. Regulation of amino acid content, fluxes, and transport through the plant is thus critical for plant adaptation to carbon and nitrogen status, development, and defence, and will be discussed in this review.

### *Amino acid metabolic pathways are branched*

Substrates for the synthesis of amino acids are mainly intermediates from glycolysis, the citric acid cycle, and the pentose phosphate pathway (Supplementary Fig. S1 at *JXB* online). Phosphoribosylpyrophosphate and erythrose-4-phosphate from the pentose phosphate pathway are the respective substrates for His and aromatic amino acid (Phe, Tyr, and Trp) synthesis. Phosphoenolpyruvate from glycolysis is the other substrate for the aromatic amino acids; pyruvate is the substrate for the branched-chain amino acid (Val and Leu) pathway and Ala synthesis; Gly, Cys, and Ser are made from 3-phosphoglycerate (also from glycolysis). Finally, the Asp-derived amino acids (Asn, Lys, Thr, Met, and Ile) are made from oxaloacetate (from the citric acid cycle), while 2-oxoglutarate is the initial metabolite for the synthesis of Glu, Gln, Pro, and Arg. The two most-studied pathways are the aromatic amino acid and Asp-derived amino acid pathways, which lead to the synthesis of amino acids that cannot be synthesized *de novo* by monogastric animals (i.e. Leu, Val, Ile, Lys, Thr, Met, Trp, and Phe). Tyr is not considered an essential amino acid, since these animals can synthesize it by Phe hydrolysis, provided that Phe levels are sufficient (see Reeds, 2000, for a discussion about the complex notion of 'essential amino acids' in humans). The Asp and the aromatic amino acid pathways are the most branched pathways, and are subject to complex regulations.

### *Amino acid metabolic pathways are compartmentalized*

Interestingly, amino acids essential for monogastric animals are synthesized in the plant chloroplast. The evolution of these pathways is complex and seems to have expanded by horizontal gene transfer from various cyanobacterial, eukaryotic, and prokaryotic sources (Reyes-Prieto and Moustafa, 2012). The aromatic amino acids Trp, Phe, and Tyr are synthesized in the chloroplast (reviewed by Maeda and Dudareva, 2012), with a cytosolic reaction leading to Phe synthesis by a tyrosine:phenylpyruvate aminotransferase (Yoo *et al.*, 2013). The enzymes for the synthesis of His, Asp-derived amino acids, and branched-chain amino acids (Leu, Ile, Val, Thr, Met, and Lys) are all addressed to the plastid (Binder, 2010; Jander and Joshi, 2010; Ingle, 2011). Most of the Arg synthesis pathway is apparently localized in the chloroplast, with some enzymes addressed to the cytosol (Slocum, 2005). The other amino acids seem to be synthesized in various compartments with different isoenzymes addressed to different organelles (Bourguignon *et al.*, 1999; Ho and Saito, 2001; Hawkesford and De Kok, 2006). Less is known about the localization of amino acid degradation. The degradation of Pro and branched-chain amino acids most likely occurs in the mitochondrion (Verslues and Sharma, 2010; Angelovici *et al.*, 2013), while the other amino acids are degraded in multiple compartments, with products heading towards the citric acid cycle in the mitochondrion. In addition to being synthesized by different pathways, amino acids are thus metabolized in different subcellular compartments. Knowledge of the compartmentation and the parallel pathways in different organelles is critical for accurate modelling of amino acid metabolism (especially for predictive purposes; Mintz-Oron *et al.*, 2012), and needs to be expanded.

## Regulation of amino acid metabolism

### *Feedback regulation of enzymes as the main control of metabolite fluxes*

Regulation of the activity of the metabolic enzymes was first elucidated by biochemical purification of the enzymes from plant tissues and subsequent characterization *in vitro* (e.g. Dotson *et al.*, 1989). Molecular cloning later enabled isolation of the corresponding cDNAs and their expression in *Escherichia coli* for precise characterization of the purified enzymes (e.g. Curien *et al.*, 2005). These approaches revealed that several enzymes are feedback regulated by the end products of various branches of the pathways, namely amino acids and *S*-adenosylmethionine (Supplementary Fig. S1). The inevitable consequence of the feedback inhibition is the toxicity of many amino acids for cells (Bonner *et al.*, 1992, 1996; Bonner and Jensen, 1997) and plant development (Voll *et al.*, 2004; Lee *et al.*, 2007; Pratelli and Pilot, 2007; Pratelli *et al.*, 2010). For instance, supplementing plants with Lys and Thr inhibits activity of the aspartate kinase (AK) of the Asp pathway (see Supplementary Fig. S1), starving the plant of Met and thereby inhibiting growth. This inhibition has been used for genetic screenings, which identified plants tolerant to toxic combinations of amino acids or amino acid analogues, and led to the identification of mutations causing enzymes to be insensitive to feedback inhibition (e.g. Bright *et al.*, 1982; Rognes *et al.*, 1983; Heremans and Jacobs, 1995; Mourad and King, 1995; Sarrobert *et al.*, 2000). Study of plants expressing the mutated genes or *E. coli* enzymes that show poor feedback sensitivity unequivocally proved the importance

of enzyme feedback inhibition for the regulation of activity of the pathways. For instance, plants expressing feedback-insensitive dihydrodipicolinate synthase (DHDPS; Shaul and Galili, 1992*a*), AK (Shaul and Galili, 1992*b*), chorismate mutase (CM; Tzin *et al.*, 2009), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS; Tzin *et al.*, 2012; Tzin *et al.*, 2013), and cystathionine-γ-synthase (CGS; Hacham *et al.*, 2006) showed an expected increase in the content of the downstream amino acids Lys, Thr, Trp, and Met, respectively. Interestingly, the content in many amino acids synthesized from other pathways was also altered in these plants, hinting at a broader regulation of the amino acid pathways, independent of the previously identified feedback regulations.

Numerous reviews have addressed the functional properties and regulation of each of the enzymes and pathways. The present review will not recapitulate these excellent reviews and will rather focus on the transcriptional regulation of the corresponding genes. The reader will find more information on each enzyme in the following reviews: the aromatic amino acid pathway (Herrmann and Entus, 2001; Tzin and Galili, 2010*a*, *b*; Maeda and Dudareva, 2012), Asp-derived amino acids (Azevedo, 2002; Azevedo *et al.*, 2006; Jander and Joshi, 2009, 2010; Joshi *et al.*, 2010; Kirma *et al.*, 2012), branched-chain amino acid pathway (Binder *et al.*, 2007; Binder, 2010), Lys (Azevedo and Lea, 2001; Galili *et al.*, 2001; Galili, 2002), Met (Amir, 2010), Ser and Gly (Bourguignon *et al.*, 1999; Ho and Saito, 2001), Arg (Verma and Zhang, 1999; Slocum, 2005), Pro (Hare *et al.*, 1999; Verma and Zhang, 1999; Lehmann *et al.*, 2010; Szabados and Savoure, 2010; Verslues and Sharma, 2010), and His (Stepansky and Leustek, 2006; Muralla *et al.*, 2007; Ingle, 2011). The regulation of asparagine and glutamine synthetases has been the subject of dedicated reviews (Cren and Hirel, 1999; Oliveira *et al.*, 2001; Gaufichon *et al.*, 2010). A summary of the identified inhibitions is presented in Supplementary Table S1 and Supplementary Fig. S1.

### *Regulation of the pathways at the transcript level*

After decades of work on the regulation of the activity of the enzymes of the Asp pathway, one could ask how far we stand from wholly understanding the regulation of this pathway. Computational modelling suggests that our present knowledge is accurate: using feedback-regulation data and the chloroplastic concentrations of amino acids (obtained by measuring the free amino acid concentration in purified chloroplasts), the model by Curien *et al.* (2009) correctly predicted steady-state fluxes of the Asp-derived amino acid pathway and *in vivo* measurements of the amino acid content in characterized mutants. Despite this encouraging result, our knowledge of the regulation of the other pathways (often split between compartments) is still scarce, and much needs to be done if one seeks to model the entire amino acid metabolism. Even if such a model is ever created, it will remain to be determined under which conditions it is valid, since modification of the abundance of the enzymes by changes in mRNA accumulation is expected to modify metabolite fluxes.

Indeed, mRNA accumulation of the transcripts encoding enzymes of the amino acid pathways has been shown to vary in response to numerous conditions. Since the first cloning of the genes encoding metabolic enzymes, numerous studies have addressed the question of the change in accumulation of the transcripts, and less often of the corresponding proteins, in response to perturbations. With the development of microarrays, systematic and more thorough analyses were performed using data mining tools, like Genevestigator (https://www.genevestigator.com/), or in-house analysis of publicly available microarray/RNAseq databases (e.g. Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/). Table 1 summarizes most of these studies. Abiotic and biotic stresses and stress hormones induce many of the genes involved in the synthesis and degradation of the aromatic amino acids, and the genes involved in the Met and *S*-adenosylmethionine pathways (Table 1). For instance, the anthranilate synthase (*AS*) gene, involved in Trp synthesis, is induced by wounding, drought, free radicals, jasmonic acid, elicitors, *Pseudomonas syringae*, and $AgNO_3$ (Zhao and Last, 1996; Zhao *et al.*, 1998; Devoto *et al.*, 2002; Catala *et al.*, 2007). The *S*-adenosylmethionine synthase (*SAMS*) gene was found to be induced by elicitors, salt stress, ethylene, and $AgNO_3$ (Schroder *et al.*, 1997; Lim *et al.*, 2002). Besides typical biotic and abiotic stresses, herbicides and amino acid treatments modify the expression of a large number of genes of these two pathways (Guyer *et al.*, 1995; Zhao *et al.*, 1998; Pasquer *et al.*, 2006; Hacham *et al.*, 2007). These data unequivocally show that the feedback inhibition of the enzymes is not the only regulatory mechanism of amino acid biosynthesis.

A broad investigation explored the changes in mRNA content of the enzymes of the amino acid metabolic pathways in response to stress, using publicly available microarray data (Less and Galili, 2008, 2009). In the first study, the authors found that the genes of the aromatic amino acid pathway were the most responsive to the stress conditions. They also concluded that, in general, the genes of the enzymes involved in degradation were the most responsive, compared with the enzymes involved in biosynthesis (Less and Galili, 2008). This result is in agreement with previous studies focused on the Lys pathway, which found that the transcript of the lysine ketoglutarate reductase/saccaropine dehydrogenase (LKR-SDH) enzyme, responsible for the first step in Lys degradation, is induced by many conditions including addition of Lys itself (Karchi *et al.*, 1995; Stepansky and Galili, 2003; Stepansky *et al.*, 2005). This regulation may explain why attempts at increasing Lys content in seeds using feedback-insensitive DHDPS and AK did not meet the expected success until the *LKR-SDH* gene was inactivated (Zhu and Galili, 2003). These results suggest that the main avenue for plant cells to control amino acid content is by controlling their degradation (Less and Galili, 2008). In the second study, using a new method to identify genes that show co-regulated expression (i.e. gene coordination), Less and Galili (2009) identified modules of genes responding in a similar fashion to sets of conditions. Three main gene modules were identified, namely an aromatic amino acid module (responding to most stresses), a Met metabolism module (positively responding to

**Table 1.** *Summary of the effect of different treatments on the expression of amino acid metabolic enzymes*

| Enzyme[a] | Pathway[b] | Sugars, light[c] | Herbicide | Amino acids | Nitrogen | Other | Biotic stresses | Abiotic stresses | Hormones[d] |
|---|---|---|---|---|---|---|---|---|---|
| AK(SDH) | AspAA | Sucrose (Zhu-Shimoni and Galili, 1998) | ↓Herbicide (Pasquer *et al.*, 2006) | | | ↓$PO_4$ (Zhu-Shimoni and Galili, 1998) | | | |
| DHDPS | Lys+ | | His herbicide (Guyer *et al.*, 1995) | | | | | | |
| LKR-SDH | Lys- | Sugars (Stepansky and Galili, 2003); (Stepansky *et al.*, 2005) | | Lys (Karchi *et al.*, 1995; Stepansky *et al.*, 2005) | ↓N (Stepansky and Galili, 2003; Stepansky *et al.*, 2005) | | | Drought (Urano *et al.*, 2009) | JA (Stepansky and Galili, 2003; Stepansky *et al.*, 2005); ABA (Stepansky and Galili, 2003; Stepansky *et al.*, 2005) |
| CGS | Met+ | Sucrose (Hacham *et al.*, 2013); light (Hacham *et al.*, 2013) | | ↓Met (Hacham *et al.*, 2007); Lys (Hacham *et al.*, 2007) | | S starvation (Nikiforova *et al.*, 2006); ↓ In mgl mutant (Joshi and Jander, 2009) | | | |
| MS | Met+ | | | | | ↓ In mgl mutant (Joshi and Jander, 2009) | | Cold, drought and salt (Narita *et al.*, 2004) | ABA (Narita *et al.*, 2004) |
| SAMS | SAM+ | | Herbicide (Pasquer *et al.*, 2006) | ↓Lys (Hacham *et al.*, 2007) | $NO_3$ (Wang *et al.*, 2001) | ↓ In mgl mutant (Joshi and Jander, 2009); polyamine (Lim *et al.*, 2002) | Elicitor (Schroder *et al.*, 1997); $AgNO_3$ (Lim *et al.*, 2002) | Salt (Schroder *et al.*, 1997; Lim *et al.*, 2002) | Ethylene (Lim *et al.*, 2002) |
| BCAT | Met- | | | | | | | Drought (Malatrasi *et al.*, 2006; Urano *et al.*, 2009) | |
| TS | Thr+ | | Glyphosate; AHAS herbicide (Zhao *et al.*, 1998) | Lys+Thr (Zhao *et al.*, 1998) | | | Elicitor (Zhao *et al.*, 1998); *Pseudomonas* and $AgNO_3$ (Zhao and Last, 1996) | Wounding (Devoto *et al.*, 2005); free radicals and ↓ heat (Zhao *et al.*, 1998) | JA (Devoto *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005; Dombrecht *et al.*, 2007) |
| TD | Ile+ | | | | | | Herbivore (Hermsmeier *et al.*, 2001) | Drought (Joshi and Jander, 2009) | JA (Samach *et al.*, 1995; Hermsmeier *et al.*, 2001) |
| AHAS | LeuVal+ | | AHAS herbicide (Zhao *et al.*, 1998) | | | | | | |
| MGL | Leu+ | | | ↓Leu (Hannah *et al.*, 2010); ↓Met (Rebeille *et al.*, 2006) | | S starvation (Goyer *et al.*, 2007) | | Drought (Joshi and Jander, 2009) | |

**Table 1.** *Continued*

| Enzyme[a] | Pathway[b] | Sugars, light[c] | Herbicide | Amino acids | Nitrogen | Other | Biotic stresses | Abiotic stresses | Hormones[d] |
|---|---|---|---|---|---|---|---|---|---|
| IMD | Leu+ | Sucrose (Jackson *et al.*, 1993) | | Leu + Thr (Jackson *et al.*, 1993) | | | | | |
| IPMS | Leu+ | | Herbicide (Pasquer *et al.*, 2006) | | | | Pathogens (De Luca *et al.*, 2011) | | |
| BCKDH | IleLeu Val- | Dark (Ishizaki *et al.*, 2005); sugars (Fujiki *et al.*, 2002) | | | | | | | |
| ETFQO | IleLeu Val- | Dark (Ishizaki *et al.*, 2005) | | | | | | | |
| IVD | IleLeu Val- | Dark (Ishizaki *et al.*, 2005); sugars (Daschner *et al.*, 2001) | | | | | | | |
| OAS | Cys+ | | | | | | | Salt (Roosens *et al.*, 1998) | JA (Sasaki-Sekimoto *et al.*, 2005) |
| SAT | Cys+ | | | | | | | | JA (Sasaki-Sekimoto *et al.*, 2005) |
| CS | PheTyr Trp+ | | | | | | Elicitor (Gorlach *et al.*, 1995) | Ozone (Janzik *et al.*, 2005; Betz *et al.*, 2009) | |
| DAHPS | PheTyr Trp+ | | His herbicide (Guyer *et al.*, 1995); glyphosate (Pinto *et al.*, 1988) | | | | Elicitor (Gorlach *et al.*, 1995); Pseudomonas (Keith *et al.*, 1991; Devoto *et al.*, 2005) | Wounding (Dyer *et al.*, 1989; Keith *et al.*, 1991); drought (Catala *et al.*, 2007); ozone (Janzik *et al.*, 2005; Betz *et al.*, 2009) | JA (Devoto *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005) |
| DHD-SDH | PheTyr Trp+ | | | | | | Elicitor (Bischoff *et al.*, 2001) | Ozone (Janzik *et al.*, 2005; Betz *et al.*, 2009) | |
| DHQS | PheTyr Trp+ | | | | | | Elicitor (Bischoff *et al.*, 1996) | Ozone (Janzik *et al.*, 2005; Betz *et al.*, 2009) | |
| EPSPS | PheTyr Trp+ | | His herbicide (Guyer *et al.*, 1995) | | | S starvation (Nikiforova *et al.*, 2006) | Elicitor (Gorlach *et al.*, 1995; Kasai *et al.*, 2005) | Ozone (Janzik *et al.*, 2005; Betz *et al.*, 2009) | |
| SK | PheTyr Trp+ | | | | | | Elicitor (Gorlach *et al.*, 1995; Kasai *et al.*, 2005) | Ozone (Betz *et al.*, 2009) | |

**Table 1.** *Continued*

| Enzyme[a] | Pathway[b] | Sugars, light[c] | Herbicide | Amino acids | Nitrogen | Other | Biotic stresses | Abiotic stresses | Hormones[d] |
|---|---|---|---|---|---|---|---|---|---|
| APT | Trp+ | | | | | | | Wounding (Devoto *et al.*, 2005) | JA (Devoto *et al.*, 2005) |
| AS | Trp+ | | His herbicide (Guyer *et al.*, 1995); glyphosate, AHAS herbicide (Guyer *et al.*, 1995; Zhao *et al.*, 1998) | Lys+Thr (Zhao *et al.*, 1998) | | S starvation (Nikiforova *et al.*, 2006) | Elicitor (Zhao *et al.*, 1998); Pseudomonas and $AgNO_3$ (Zhao and Last, 1996) | Free radicals and ↓ heat (Zhao *et al.*, 1998); wounding (Devoto *et al.*, 2005); drought (Catala *et al.*, 2007) | JA (Devoto *et al.*, 2005) |
| IGPS | Trp+ | | | | | | | Wounding (Devoto *et al.*, 2005) | JA (Devoto *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005; Dombrecht *et al.*, 2007) |
| PAI | Trp+ | | Herbicide (Pasquer *et al.*, 2006) | | $NO_3$ (Wang *et al.*, 2001) | | Elicitor (Zhao and Last, 1996); $AgNO_3$ (He and Li, 2001) | Wounding (Dyer *et al.*, 1989; Keith *et al.*, 1991); drought (Catala *et al.*, 2007) | |
| TMO | Trp- | | | | | | | | JA (Dyer *et al.*, 1989) |
| CM | PheTyr+ | | His herbicide (Guyer *et al.*, 1995) | | | | *Pseudomonas* (Mobley *et al.*, 1999) | Wounding (Dyer *et al.*, 1989; Mobley *et al.*, 1999; Devoto *et al.*, 2005); ozone (Betz *et al.*, 2009) | JA (Devoto *et al.*, 2005); GA (Xu *et al.*, 2001) |
| PAT | PheTyr+ | | His herbicide (Guyer *et al.*, 1995); glyphosate and AHAS herbicide (Guyer *et al.*, 1995; Zhao *et al.*, 1998) | Lys + Thr (Zhao *et al.*, 1998) | | | *Pseudomonas* and $AgNO_3$ (Zhao and Last, 1996) | | |
| TAT | Phe+ | | | | | | Coronatine (Lopukhina *et al.*, 2001) | Wounding (Titarenko *et al.*, 1997) | JA (Titarenko *et al.*, 1997; Lopukhina *et al.*, 2001); ABA (Titarenko *et al.*, 1997) |

**Table 1.** *Continued*

| Enzyme[a] | Pathway[b] | Sugars, light[c] | Herbicide | Amino acids | Nitrogen | Other | Biotic stresses | Abiotic stresses | Hormones[d] |
|---|---|---|---|---|---|---|---|---|---|
| PAL | Phe- | | Herbicide (Pasquer *et al.*, 2006) | | $NO_3$ (Wang *et al.*, 2001) | | Elicitor (Gorlach *et al.*, 1995) | Wounding (Dyer *et al.*, 1989; Keith *et al.*, 1991); drought (Catala *et al.*, 2007) | |
| TDC | Tyr- | | | | | | Coronatine (Lehmann and Pollmann, 2009) | ↓Salt (Lehmann and Pollmann, 2009); wounding (Lehmann and Pollmann, 2009) | JA (Lehmann and Pollmann, 2009) |
| ASSY | Arg+ | | ↓Herbicide (Pasquer *et al.*, 2006) | | | | | | |
| δOAT | Arg+ | | | | | | | Salt (Roosens *et al.*, 1998) | |
| ADC | Arg- | | | | | | | Drought (Urano *et al.*, 2009) | |
| P5CS | Pro+ | | | | | | | Drought (Urano *et al.*, 2009) | |
| PRODH | Pro- | | | ↓Leu (Hannah *et al.*, 2010) | | | | | |
| HDH | His+ | | His herbicide (Guyer *et al.*, 1995) | | | | | | |
| ASN | AsmIt | Dark (Herrera-Rodriguez *et al.*, 2004); sugar starvation (Chevalier *et al.*, 1996); low $CO_2$ (Herrera-Rodriguez *et al.*, 2004) | | ↓AA (Chevalier *et al.*, 1996); Gln (Kawachi *et al.*, 2002) | $NH_4$ (Chevalier *et al.*, 1996; Kawachi *et al.*, 2002; Herrera-Rodriguez *et al.*, 2004); $NO_3$ (Wang *et al.*, 2001) | | *Pseudomonas* (De Luca *et al.*, 2011) | Salt (Chevalier *et al.*, 1996) | |
| ASN1 | AsmIt | ↓Sucrose (Oliveira *et al.*, 2001); ↓light (Oliveira *et al.*, 2001); dark (Lam *et al.*, 1998; Wong *et al.*, 2004) | | | Low C/N (Lam *et al.*, 1998) | | | | |

**Table 1.** *Continued*

| Enzyme[a] | Pathway[b] | Sugars, light[c] | Herbicide | Amino acids | Nitrogen | Other | Biotic stresses | Abiotic stresses | Hormones[d] |
|---|---|---|---|---|---|---|---|---|---|
| ASN2 | AsmIt | light (Lam *et al.*, 1998) | | | High C/N (Lam *et al.*, 1998); NH4 (Wong *et al.*, 2004); $NO_3$ (Wang *et al.*, 2000; Wang *et al.*, 2003) | | | Salt (Wong *et al.*, 2004) | |
| GOGAT | AsmIt | | | | $NO_3$ (Wang *et al.*, 2000; Dombrecht *et al.*, 2007) | | | | |
| GS | AsmIt | | | | $NH_4$ (Kawachi *et al.*, 2002); $NO_3$ (Wang *et al.*, 2000; Wang *et al.*, 2003) | | | | |
| GS1 | AsmIt | Sucrose (Oliveira *et al.*, 2001); light (Oliveira *et al.*, 2001) | Glyphosate and AHAS herbicide (Zhao *et al.*, 1998) | Gln, Asp, Glu (Oliveira *et al.*, 2001) | | | Elicitor (Zhao *et al.*, 1998); *Pseudomonas* (Olea *et al.*, 2004) | Free radicals (Zhao *et al.*, 1998) | |
| GS2 | AsmIt | | ↓ Herbicide (Guyer *et al.*, 1995) | | | | | | |
| GAD | AsmIt | | | | $NO_3$ (Wang *et al.*, 2001) | | | Drought (Urano *et al.*, 2009) | |

[a] Enzyme abbreviations are detailed in Supplementary Table 1. The isoform is specified when known (ASN1, ASN2, GS1, GS2).
[b] Amino acid three-letter code means the pathway of the corresponding amino acid(s); a '-' after the amino acid name means degradation pathway, a '+' after the amino acid name means biosynthesis pathway. AspAA, Asp-derived amino acids (Lys, Thr, Met, Ile); SAM, *S*-adenosylmethionine; AsmIt, assimilation pathway (Glu, Gln, Asp, Asn).
[c] Expression changes are increase in mRNA accumulation, except when ↓ is placed before the condition, meaning decrease.
[d] JA, Jasmonic acid; ABA abscisic acid; $NO_3$, nitrate; $NH_4$, ammonium; N, nitrogen.

conditions of active growth), and a catalytic module (induced by most stresses and repressed by active growth). The existence of these modules suggests that a common signalling and regulation mechanism exists and controls the expression of genes involved in the same or different pathways. Common transcription factors are expected to be at play, a hypothesis tested by co-expression analysis of metabolic enzymes and transcription factor genes (Joshi *et al.*, 2010). Co-expression was detected between these groups of genes, setting the ground for more detailed research.

Characterization of the role of different members of the basic leucine zipper (bZIP) transcription factor family showed that, in response to stress, the induction of *AtASN1* and *AtProDH* (synthesis of Asn and degradation of Pro, respectively) by low-carbon conditions is mediated by transcription factors bZIP1, -53, and -11 (Hanson *et al.*, 2008; Kang *et al.*, 2010; Obertello *et al.*, 2010; Dietrich *et al.*, 2011). bZIPs (bZIP12/DPBF4, bZIP39/ABI5) can also have inhibitory roles, downregulating the expression of AK during darkness and low-sugar conditions (Ufaz *et al.*, 2011). Interestingly, the expression of *bZIP1* and *bZIP39* is regulated by sugars (Kang *et al.*, 2010) and abscisic acid (ABA) (Brocard *et al.*, 2002), respectively, linking amino acid metabolism to sugar signalling and the stress hormone ABA. Plant hormones have been shown to be involved in the control of metabolism in general, notably with an effect of cytokinins on nitrogen metabolism (reviewed by Sakakibara *et al.*, 2006; Rubio *et al.*, 2009). Several transcription factors have also been shown to control the expression of *DAHPS*, 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*), *CM*, and phenylalanine ammonia lyase (*PAL*), involved in Phe synthesis and degradation. An increase in the content of phenylpropanoid metabolites, part of the secondary metabolism downstream from Phe, has always been detected with a concomitant increase in the activity of the upstream Phe pathway. In good agreement with this observation, most of the transcription factors that have been shown to regulate the expression of the genes of the phenylpropanoid pathway also control, maybe indirectly, the expression of genes of the aromatic amino acid pathway (reviewed by Maeda and Dudareva, 2012; Tzin and Galili, 2010*b*). These data show that complex networks have to be expected for the regulation of amino acid homeostasis, since signals from nitrogen and carbon and demand for secondary compounds within the same organ or from other organs have to be integrated to deliver an optimal amino acid synthesis rate.

Regulation at a post-transcriptional/translational level has been described for δ-pyrroline-5-carboxylate reductase (P5CR) and CGS. P5CR is involved in Pro synthesis in response to stress, and CGS in Met synthesis. An intriguing discrepancy between *P5CR* mRNA and protein contents prompted the study of the translation efficiency of the *P5CR* mRNA under salt stress (Hare *et al.*, 1999). The authors found that the 5′-untranslated region of *P5CR* is involved in translation inhibition and in concomitant mRNA stabilization, and surprisingly in the control of transcription efficiency (Hua *et al.*, 2001). The meaning of these partly opposing directions of regulation is not completely understood. *CGS* mRNA degradation and translation is controlled by *S*-adenosylmethionine. *S*-adenosylmethionine binds the nascent CGS protein at the freshly translated so-called MTO domain and leads to ribosome stalling, causing decreased mRNA accumulation and protein synthesis (Onouchi *et al.*, 2005). A secondary *CGS* transcript has been detected in plants that lacks the MTO region and that is not subjected to inhibition by *S*-adenosylmethionine (Hacham *et al.*, 2006). It is supposed that the shorter form of the *CGS* transcript is formed from the full-length transcript by cleavage of the mRNA region encoding the MTO domain, which probably forms a hairpin (Hacham *et al.*, 2006). Natural production of this transcript would allow Met synthesis even in presence of high Met and *S*-adenosylmethionine concentrations.

### *Different protein modifications are likely to be involved in regulation of the pathways*

Little is known about the post-translational modifications of enzymes of the amino acid biosynthetic pathways or the effects of interactions with other proteins. A proteomics approach identified four enzymes of the Met cycle, namely adenosylhomocysteinase/*S*-homocysteine hydrolase (SAHH), *O*-acetylserine (thiol) lyase (OAS), SAMS, and methionine synthase (MS) as proteins that could be nitrosylated in plants (Lindermayr *et al.*, 2005). Further work showed that nitrosylation of a Cys close to the active site of AtSAMS1 reduced its activity by about 60%. The physiological relevance of this modification is not clear at present, since the two other tested SAMS (AtSAMS2 and AtSAMS3) were not nitrosylated (Lindermayr *et al.*, 2006). Phosphoproteomics approaches have uncovered the diversity of proteins that can be phosphorylated. Results from experiments deposited in the PhosphAt database (Heazlewood *et al.*, 2008; Durek *et al.*, 2010) showed that about one-third of the *Arabidopsis* proteins involved in amino acid metabolism are phosphorylated. These results are in agreement with the early report of the importance of phosphorylation for the induction of LKR activity in response to Lys treatment in tobacco (Karchi *et al.*, 1995). LKR phosphorylation is triggered by resupply of nitrate after starvation (PhosphAt database) and *in vitro* studies have found that the activity of LKR-SDH is decreased by dephosphorylation (Miron *et al.*, 1997; Zhu *et al.*, 2002).

Phosphorylation has been shown to affect the binding of 14-3-3 proteins, involved in the regulation of the activity of many proteins, to glutamine synthetase (GS) (Moorhead *et al.*, 1999; Shin *et al.*, 2011) and nitrate reductase (Bachmann *et al.*, 1996). Based on these results, Diaz *et al.* (2011) characterized the metabolic changes due to overexpression of 14-3-3 proteins in *Arabidopsis*. The authors found that overexpression of some 14-3-3s led to the modification of the activity of GS, SDH, glutamate dehydrogenase (GDH), and Asp transaminase, as well as many enzymes of carbohydrate metabolism (Diaz *et al.*, 2011). Similarly, a proteomics approach showed that 14-3-3 proteins bind to AtBCAT1 (branched-chain amino acid pathway), AtDAHPS1, AtDHAPS2 (aromatic amino acid pathway), and AtOAS (Cys pathway) (Chang *et al.*, 2009), suggesting that 14-3-3 proteins are involved in

the central activity of key enzymes of amino acid synthesis. Protein activity is also controlled by protein degradation rate, especially through the ubiquitin–proteasome system (Vierstra, 2009). A yeast two-hybrid screening recently identified three Kelch motif F-box proteins as interacting partners of *Arabidopsis* PAL1 and PAL2 (Zhang *et al.*, 2013*c*). These F-box proteins, part of the E3 ubiquitin ligase SCF complex, control the activity of the two *Arabidopsis* PAL proteins by mediating their ubiquitination and subsequent degradation, therefore controlling the flux towards the synthesis of phenylpropanoids (Zhang *et al.*, 2013*c*).

### *Coordination of the pathways*

Several observations suggest that the activities of the amino acid pathways are coordinated at the protein level. The activity of AK-SDH (first enzyme in the Asp amino acid pathway) is controlled by amino acids from other pathways, namely Ala, Ser, Leu, Ile, and Val (Curien *et al.*, 2005; Supplementary Fig. S1), and the activity of ornithine-δ-aminotransferase (δOAT), involved in ornithine degradation, is inhibited by Ser, Leu, and Val (Sekhar *et al.*, 2007; Supplementary Fig. S1). While the importance of these regulations has not been assessed *in vivo*, it is likely that these regulations are not unique and that others will be identified in the near future.

Several reactions involving products or intermediaries link branches of various pathways. For instance, Phe can be synthesized from Tyr to maintain a balance between Phe and Tyr in the cytosol (Yoo *et al.*, 2013). Glu is required for transamination reactions for the synthesis of most amino acids [e.g. mediated by branched-chain amino acid aminotransferase 3 (BCAT3) for the branched-chain amino acids; diaminopimelate amino transferase for Lys; histidinol-phosphate aminotransferase for His; and prephenate amino transferase (PAT) for Phe and Tyr], while Gln is used for the synthesis of anthranilate from chorismate (mediated by AS). Carbon skeleton or chemical groups of some amino acids are used for synthesis of other amino acids (e.g. Ser is used for the synthesis of Trp, Cys for the synthesis of Met, and Asp for the synthesis of Arg). These direct connections between pathways imply that the donor metabolites are synthesized coordinately to their use in the other pathways. Interestingly, a recently described reaction links the Asp-derived amino acid pathway and the aromatic amino acid pathway to the regulation of the synthesis of auxin (from Trp) and ethylene (from Met) by the aminotransferase VAS1 (Zheng *et al.*, 2013).

Genetic modification of the activity of the pathways by gene knockout or overexpression of feedback-inhibited enzymes led to surprising discoveries concerning the accumulation of amino acids in the plant. For instance, plants expressing a mutant version of arogenate dehydratase (Phe biosynthesis) or two decarboxylases specific for Trp or Tyr showed modification of the content in amino acids from most of the other pathways (Guillet *et al.*, 2000; Huang *et al.*, 2010). Expression of feedback-insensitive AK (Asp pathway), CGS (Cys biosynthesis), threonine aldolase (Thr degradation) or a combination of feedback-insensitive DHDPS and knockdown LKR-SDH (Lys metabolism) modified the content in most of the amino acids, in addition to the originally targeted amino acid(s) (Heremans and Jacobs, 1995; Jander *et al.*, 2004; Zhu and Galili, 2004; Hacham *et al.*, 2008). Finally, knockout of the isovaleryl-CoA dehydrogenase gene (*IVD*; branched-chain amino acid degradation) led to an increase in most of the amino acid accumulation in seeds (Gu *et al.*, 2010). This set of observations suggests that disturbance of one pathway has repercussions on the activities of the other pathways, which cannot easily be explained by feedback inhibitions only (Zhu and Galili, 2003). In contrast, increasing His and Trp content was shown to have little or no consequence on the accumulation of amino acids from other pathways (Ingle *et al.*, 2005; Wakasa *et al.*, 2006; Tzin *et al.*, 2012). Predicting the effect of artificial changes in the expression of metabolic genes on the amino acid content of plants thus appears almost impossible, greatly limiting our ability for metabolic engineering (Galili and Amir, 2012).

Three observations led to the hypothesis of the existence of cross-regulation of amino acid metabolic pathways at the transcriptional level: the expression of enzymes from various pathways was modified in response to an inhibitor of His synthesis (Guyer *et al.*, 1995), an inhibitor of the branched-chain amino acid synthesis (Zhao *et al.*, 1998; Pasquer *et al.*, 2006), and addition of amino acids in the growth medium (e.g. addition of Thr and Lys, inhibiting Met synthesis) (Jackson *et al.*, 1993; Zhao *et al.*, 1998). Microarray analyses have studied the effect of perturbation of amino acid synthesis caused by treating *Arabidopsis* plants with the herbicide compound imidazolinone, a blocker of acetohydroxyacid synthase (AHAS; branched-chain amino acids; Manabe *et al.*, 2007; Das *et al.*, 2010), or by mutation of amino acid metabolic enzymes, namely threonine deaminase/dehydratase TD (*omr1*, Ile synthesis; Yu *et al.*, 2013), desulfhydrase (*des1*, Cys degradation; Alvarez *et al.*, 2010), OAS (*oas-a1*, Cys synthesis; Alvarez *et al.*, 2010), and glutamate synthase (GOGAT) (*glu1-2*, Glu synthesis; Kissen *et al.*, 2010). These perturbations were all reported to affect amino acid content, as well as the expression of genes responding to abiotic stresses (drought, salt, and heat), or to be involved in plant immunity to pathogens (Manabe *et al.*, 2007; Alvarez *et al.*, 2010; Das *et al.*, 2010; Kissen *et al.*, 2010; Yu *et al.*, 2013). In contrast, treating plants with glyphosate, a blocker of the EPSPS enzyme from the shikimate pathway, led to very little change at the metabolic and gene expression levels after 24 h. Changes in gene expression could nevertheless be detected several days after glyphosate treatment (Das *et al.*, 2010; Table 1), in good agreement with the fact that modifications of the activity of the aromatic amino acid pathway has few consequences on the accumulation of other amino acids (see above). These data suggest that alterations in the activity of specific amino acid pathways lead to a stress response, which has been shown in turn to modify broader metabolic activity (Hey *et al.*, 2010). It is thus possible that the apparent cross-regulation of the pathways is the consequence of a stress response, triggered by amino acid perturbation, as postulated previously (Denby and Last, 1999), and not the consequence of a dedicated process. Triggering of the stress response would then explain some developmental defects, such as reduced growth, observed following

overaccumulation of Lys and Met in plants (Frankard *et al.*, 1992; Hacham *et al.*, 2006), and would impede our ability to engineer plants with altered amino acid metabolism.

## Amino acid transport in plants

### *Importance of transporters for metabolism*

A reconstruction of yeast metabolism showed that 401 of its 1312 distinct biochemical reactions correspond to membrane transport steps (Herrgard *et al.*, 2008; Heavner *et al.*, 2013). Similarly, in a model of *Arabidopsis* central metabolism, 772 transport steps were predicted to allow the 1363 biochemical reactions of this model to occur (Mintz-Oron *et al.*, 2012). Our current knowledge about the identity of the transporters mediating these steps is limited, and only a handful of intracellular transporters is known (Linka and Weber, 2010). While co-expression analyses recently helped to identify some of these transporters [e.g. PLGG1, a plastidic glycolate glycerate transporter (Pick *et al.*, 2013); reviewed by Bordych *et al.*, 2013], much is left to be done to get the whole set of transporters involved in metabolism. This gap in our knowledge is well examplified by amino acid metabolism: amino acid synthesis pathways are compartmented, and transport between various intracellular compartments (chloroplast, mitochondrion, peroxisome, and vacuole) and the cytosol is essential for metabolic activity. In addition, long-distance transport of amino acids in the plant, involving phloem or xylem loading and unloading, uptake from the soil, and transfer to the embryo, also requires several steps of inward and outward transport across membranes. Our current knowledge about plant amino acid transporters is summarized below, focusing on the regulation of their activity in response to changing growth conditions.

### *Identity of the plant amino acid transporters*

The *Arabidopsis* genome is anticipated to contain about 100 genes encoding amino acid transporters. They belong to the amino acid–polyamine–choline (APC) transporter superfamily (Jack *et al.*, 2000), and the UMAMIT family (part of the DMT superfamily; Jack *et al.*, 2001).

The APC superfamily encompasses five families (Jack *et al.*, 2000). Out of these five families, four were shown to mediate amino acid transport: the APC family, the AAAP (auxin/amino acid permease) family, the alanine or glycine: cation symporter (AGCS) family, the cation–chloride cotransporter (CCC) family, and the hydroxy/aromatic amino acid permease (HAAAP) family. Of main interest are the plant transporters of the APC and AAAP families, gathering, respectively, the cationic amino acid transporters (CATs), amino acid/choline transporters (ACTs), and polyamine $H^+$-symporters (PHSs) in the former, and amino acid permeases (AAPs), lysine and histidine transporters (LHTs), proline transporters (ProTs), γ-aminobutyric acid transporters (GATs), auxin transporters (AUXs), and aromatic and neutral amino acid transporters (ANTs) in the latter. Finally, a phylogenetic tree of the APC superfamily also includes, on a branch of its own (Fischer *et al.*, 1998), the distantly related transporter AtBAT1/AtGABP (Dundar and Bush, 2009; Michaeli *et al.*, 2011).

Most of the APC superfamily amino acid transporters that were thoroughly characterized show importer properties (Tegeder and Rentsch, 2010), i.e. they mediate transport of amino acids towards the cytosol, and are mainly involved in long-distance transport. However, the question remains open as to whether all members of this superfamily are importers. This is probably not the case, since AtBAT1/AtGABP was described as a bidirectional transporter (Dundar and Bush, 2009), mediating γ-aminobutyric acid (GABA) transport to the mitochondrion (Michaeli *et al.*, 2011). However, since the AtBAT1/GABP genomic sequence is very divergent from the other APC superfamily members, it may be assumed that its unusual functional properties reflect this sequence divergence and may not be widespread within the superfamily. Transporters with strict export properties are still missing, despite physiological evidence for such an activity (reviewed by Okumoto and Pilot, 2011), and a thorough characterization of a facilitator was reported only recently. Similar to AtBAT1/GABP, AtSiAR (AtUMAMIT18) also displays bidirectional transport properties (Ladwig *et al.*, 2012). The UMAMIT family belongs to the DMT superfamily, which otherwise mainly comprises transporters for triose phosphate and nucleotide–sugar compounds (Jack *et al.*, 2001). Only two members of this family have been characterized so far: WAT1/AtUMAMIT05 mediating auxin transport (Ranocha *et al.*, 2013), and AtSiAR1/AtUMAMIT18 (Ladwig *et al.*, 2012) mediating amino acid transport, which suggests a spectrum of substrates as broad as that of the APC superfamily transporters.

### *Regulation of transporters at the transcript level*

The expression pattern, mutant phenotype, and functional properties, when available, of all characterized amino acid transporters have been reviewed in depth elsewhere (Tegeder and Rentsch, 2010; Tegeder, 2012, 2014). Orthologues of *Arabidopsis* transporters have been identified and investigated in several other plants, including *Brassica napus*, *Solanum tuberosum*, *Lotus japonicus*, *Lycopersicon esculentum*, and *Hordeum vulgare*. A recent genome-wide survey identified 85 amino acid transporters in rice (Zhao *et al.*, 2012). Since so few amino acid transporters are fully characterized, information about the mechanisms that regulate their expression is even scarcer. To date, there have been only 23 reports providing data about abiotic or biotic stresses (developmental regulation being outside the scope of this review) that may affect amino acid transporters expression. These are listed in Table 2.

Except for the recent expression investigation in rice (Zhao *et al.*, 2012) no large-scale analysis of the factors that affect amino acid transporter expression has been performed in *Arabidopsis* or any other plant. Among the data published so far, most of the changes in transcript levels were found to be associated with biotic stress such as nematode wounding (Hammes *et al.*, 2006; Elashry *et al.*, 2013; Marella *et al.*,

**Table 2.** *Summary of the effect of different treatments on the expression of amino acid transporters*

| Family | Gene | Induction by | References | Repression by | References |
|---|---|---|---|---|---|
| AAP | AtAAP1 | Nematodes | Elashry *et al.*, 2013 | | |
| | | Nitrogen | Ortiz-Lopez *et al.*, 2000; Guo, 2004; Liu and Bush, 2006 | | |
| | | Sugars | Ortiz-Lopez *et al.*, 2000; Guo, 2004 | | |
| | | Amino acids | Guo, 2004 | | |
| | | Light | Ortiz-Lopez *et al.*, 2000 | | |
| | AtAAP2 | Nematodes | Elashry *et al.*, 2013 | | |
| | | Amino acids | Guo, 2004 | | |
| | AtAAP3 | Nematodes | Elashry *et al.*, 2013; Marella *et al.*, 2013 | | |
| | AtAAP4 | Nematodes | Elashry *et al.*, 2013 | Drought, salt | Rentsch *et al.*, 1996 |
| | AtAAP5 | | | Nematodes | Elashry et al 2013 |
| | AtAAP6 | Nematodes | Elashry *et al.*, 2013; Marella *et al.*, 2013 | Drought, salt | Rentsch *et al.*, 1996 |
| | | | | Amino acids | Guo, 2004 |
| | AtAAP7 | | | Nematodes | Elashry *et al.*, 2013 |
| | AtAAP8 | Nematodes | Elashry *et al.*, 2013 | In *aap1* seeds 16–18 DAF | Sanders *et al.*, 2009 |
| | | In *aap1* seeds | Sanders *et al.*, 2009 | In *aap2* | Zhang *et al.*, 2010 |
| | BnAAP1 | Nitrogen | Tilsner *et al.*, 2005 | | |
| | BnAAP2 | Nitrogen | Tilsner *et al.*, 2005 | | |
| | BnAAP6 | Nitrogen | Tilsner *et al.*, 2005 | | |
| | VfAAP1 | | | Amino acids, sugars | Miranda *et al.*, 2001 |
| | OsAAP4 | | | Drought, salt, cold | Zhao *et al.*, 2012 |
| | OsAAP5 | Drought, salt | Zhao *et al.*, 2012 | | |
| | OsAAP6 | Drought, salt | Zhao *et al.*, 2012 | | |
| | OsAAP8 | | | Drought, salt, cold | Zhao *et al.*, 2012 |
| | OsAAP11 | Drought, salt | Zhao *et al.*, 2012 | | |
| | OsAAP13 | Drought, salt | Zhao *et al.*, 2012 | | |
| | OsAAP15 | Drought, salt, cold | Zhao *et al.*, 2012 | | |
| | PsAAP1 | Nitrogen, amino acids, light | Tegeder *et al.*, 2007 | Dark | Tegeder *et al.*, 2007 |
| | PsAAP2 | Nitrogen, amino acids, light | Tegeder *et al.*, 2007 | Dark | Tegeder *et al.*, 2007 |
| ProT | AtProT2 | Drought | Rentsch *et al.*, 1996; Grallath *et al.*, 2005 | | |
| | | Salt | Rentsch *et al.*, 1996 | | |
| | | Wounding | Grallath *et al.*, 2005 | | |
| | | Nitrogen | Liu and Bush, 2006 | | |
| | Ab/ProT1 | Salt | Waditee *et al.*, 2002 | | |
| | Ab/ProT2 | Salt | Waditee *et al.*, 2002 | | |
| | Ab/ProT 3 | Salt | Waditee *et al.*, 2002 | | |
| | HvProT1 | Salt | Ueda *et al.*, 2008 | | |
| | LePRoT3 | Drought, salt | Schwacke *et al.*, 1999 | | |
| | McAAT1/ | | | | |
| ProT | Salt | Popova *et al.*, 2003 | | | |
| | OsProT3 | | | Drought, salt | Zhao *et al.*, 2012 |
| CAT | AtCAT2 | | | In *aap2* | Zhang *et al.*, 2010 |
| | AtCAT6 | Nematodes | Hammes *et al.*, 2006 | | |
| | | In *aap1* seeds | Sanders *et al.*, 2009 | | |
| | OsCAT6 | Drought | Zhao *et al.*, 2012 | | |
| LHT | AtLHT1 | | | Nematodes | Elashry *et al.*, 2013 |
| | | Amino acids | Hirner *et al.*, 2006 | | |
| | | Nitrogen | Hirner *et al.*, 2006; Liu and Bush, 2006 | | |
| | | Pathogen attack | Liu *et al.*, 2010 | | |
| | | In *aap2* | Zhang *et al.*, 2010 | | |
| | LjLHT1 | Mycorhyzal fungi | Guether *et al.*, 2011 | | |
| | McAAT2/ | | | | |
| LHT | Salt | Popova *et al.*, 2003 | | | |

**Table 2.** *Continued*

| Family | Gene | Induction by | References | Repression by | References |
|---|---|---|---|---|---|
| ANT | PgLHT | Hormones, salt, amino acids | Zhang *et al.*, 2013*b* | | |
| | AtANT1 | Nitrogen | Liu and Bush, 2006 | In *aap2* | Zhang *et al.*, 2010 |
| | OsANT3 | Drought, cold, salt | Zhao *et al.*, 2012 | | |
| | OsANT4 | Salt | Zhao *et al.*, 2012 | | |
| GAT | AtGAT1 | Wounding | Meyer *et al.*, 2006 | | |
| | OsGAT2 | Drought | Zhao *et al.*, 2012 | | |
| ACT | OsBAT4 | | | Drought, cold, salt | Zhao *et al.*, 2012 |
| | OsBAT7 | Salt | Zhao *et al.*, 2012 | | |
| VAAT / ATL | OsATL6 | Drought, cold, salt | Zhao *et al.*, 2012 | | |
| | OsATL9 | | | Drought | Zhao *et al.*, 2012 |
| | OsATL11 | Salt | Zhao *et al.*, 2012 | | |
| | OsATL13 | Drought, salt | Zhao *et al.*, 2012 | | |

2013), pathogen attack (Liu *et al.*, 2010), or interaction with mycorrhizal fungi (Guether *et al.*, 2011), most likely reflecting the interplay of aggressors trying to highjack nitrogenous compounds, possibly by using the plant's own transporters, and plants trying to retain or redistribute these compounds. Another interesting aspect is the effect of nitrogenous or carbon metabolites on the expression of amino acid transporters. *AtAAP1*, *AtProT2*, *AtLHT1*, and *AtANT1*, belonging to different subfamilies, displayed an increased expression upon high $NO_3$ treatment (Liu and Bush, 2006). The authors also refer to a study showing that AAP family members are affected by metabolites (Guo, 2004): *AtAAP1* is upregulated by glucose, sucrose, $NH_4$, and amino acids, and *AtAAP2* is upregulated by glutamate, whereas *AtAAP6* is downregulated by glutamine. These modifications in *AtAAP1* expression are in agreement with a previous investigation focusing on the effects of light, sugar, and nitrogen starvation and resupply (Ortiz-Lopez *et al.*, 2000). These results suggest the presence of an integrative mechanism that adapts amino acid transporter expression to the availability of organic and inorganic nitrogen sources and photosynthetic activity. It is interesting to note that, in *B. napus*, *BnAAP1*, *-2* and *-6* are upregulated in flowers upon high nitrogen supply (Tilsner *et al.*, 2005), whereas *VfAAP1* is downregulated by the combined effect of high glutamine and sucrose or 1 mM cysteine (Miranda *et al.*, 2001). The only report on how hormones may affect amino acid transporter expression was performed in *Panax ginseng*, where *PgLHT1* expression was increased in response to ABA, salicylic acid, and methyl jasmonate (Zhang *et al.*, 2013*b*). Among the environmental factors that were investigated, drought, cold, light, and salt stress were shown to affect the expression of members of all the amino acid transporter families in several plants, notably rice (Rentsch *et al.*, 1996; Schwacke *et al.*, 1999; Ortiz-Lopez *et al.*, 2000; Waditee *et al.*, 2002; Popova *et al.*, 2003; Ueda *et al.*, 2008; Zhao *et al.*, 2012). Finally, amino acid transporter expression may be modified in response to genetic alteration: *AtCAT6* and *AtAAP8* transcript levels were modified in the seeds of *aap1* knockout mutants compared with the wild type (Sanders *et al.*, 2009). *AtAAP8*, *AtCAT2*, and *AtANT1* were also downregulated in *aap2* T-DNA insertion lines (Zhang *et al.*, 2010), suggesting that a master regulation mechanism adjusts the expression and activity of amino transporters in an integrative fashion.

### *Can transporters be regulated at the protein level?*

The post-translational regulation of amino acid transporters has not yet been documented in plants. Ubiquitination is a major control mechanism of protein activity in fungi, plants, and animals, and membrane proteins are no exception to this biological phenomenon. Ubiquitination has been shown to alter activity, abundance, localization, and function of various membrane proteins (MacGurn *et al.*, 2012). In mammals, among others, cytokine and interferon receptors, various channels and transporters and G protein-coupled receptors have been shown to be the target of ubiquitin-mediated regulation (for reviews, see Hicke and Dunn, 2003; Staub and Rotin, 2006). Receptor tyrosine kinase downregulation serves as the major negative feedback regulatory mechanism of receptor signalling, and this downregulation is mostly mediated by receptor ubiquitination (Miranda and Sorkin, 2007). Of special interested for the present review is the report that the sodium-dependent neutral amino acid transporter ATA2 is downregulated after polyubiquitination by the ligase Nedd4 in mammals (Hatanaka *et al.*, 2006). Ubiquitination-mediated amino acid transporter regulation was also demonstrated in yeast, with the two Trp permeases ScTat1 and ScTat2 regulated by the ubiquitin ligase ScRsp5, triggering endocytosis and endocytic degradation (Beck *et al.*, 1999; Suzuki *et al.*, 2013). The transporters Lys permease ScLyp1, Arg permease ScCan1, Met permease ScMup1, and Glu permease ScDip5 are also degraded after arrestin-mediated ubiquitination in response to specific amino acids or environmental stresses (for a review, see Leon and Haguenauer-Tsapis, 2009; Becuwe *et al.*, 2012). Finally,

the general amino acid permease ScGap1 undergoes post-endocytic targeting to the vacuole upon ubiquitination by ScRsp5 in presence of $NH_4^+$ (Springael *et al.*, 1999).

Evidence for nutrient transporter regulation by ubiquitination in plants is much more scarce but is increasing. Ubiquitination causes boron transporter 1 (BOR1) to be internalized and degraded (Kasai *et al.*, 2011) and modulates the activity of iron-regulated transporter 1 (IRT1; Barberon *et al.*, 2011). Although ubiquitination-mediated amino acid transporter regulation is still not strictly documented in plants, it is tempting to consider that the *Arabidopsis* glutamine dumper 1 (GDU1) protein is likely to be involved in such a mechanism. The overexpression of this single transmembrane domain protein results in small plants displaying an increased amino acid content throughout the plant (Pilot *et al.*, 2004) and increased amino acid export activity (Pratelli *et al.*, 2010). GDU1 interacts with and is ubiquitinated by the E3 ubiquitin ligase LOG2, and this interaction is necessary for the Gdu1D phenotype (Pratelli *et al.*, 2012). Although the role of GDU1 is still not elucidated, it is hypothesized that it is an adaptor that brings together LOG2 and a yet-to-be discovered amino acid exporter, allowing the ubiquitin ligase to regulate the activity of the transporter.

Phosphorylation is another common post-translational modification known to alter protein activity. The only evidence for amino acid transporter phosphorylation in plants comes from the PhosphAt database, where phosphorylation sites for four transporters are reported (CAT4, CAT8, LHT4, and VAAT4). These sites have been found to be phosphorylated by proteomics approaches, but no specific study has yet focused on the role of these phophorylations. However, in the germinating barley grain, the peptide transporter HvPTR1 is phosphorylated in response to rising amino acid levels resulting from reserve breakdown, leading to rapid inhibition of its activity (Waterworth *et al.*, 2005). In contrast, the activity of the mammalian amino acid transporter EAAT5 is stimulated upon phosphorylation by kinases SGK1 and SGK3, which increased cell-surface abundance of the carrier (Boehmer *et al.*, 2005). Similar effects were observed for the excitatory amino acid carrier 1 (EAAC1), which is upregulated by various protein kinase C subtypes (Gonzalez *et al.*, 2002). In mammalian cells, phosphorylation of amino acid and related neurotransmitter transporters seems to affect trafficking to and from the plasma membrane, and in some cases raft association or dissociation, thereby adapting activity to the cell's needs (summarized by Samluk *et al.*, 2010). The mechanism of membrane removal of the human cationic amino acid transporter Hs CAT1 upon protein kinase C activation was further dissected and ubiquitination-triggered, clathrin-mediated endocytosis was shown to result in transport activity inhibition (Vina-Vilaseca *et al.*, 2011).

## Coordination of metabolism and transport activities

While transport and enzymatic activities need to be coordinated to enable supply of all compartments of the cell with amino acids, co-regulation of metabolism and transport has not been investigated directly so far. Nevertheless, examination of published studies revealed that amino acid enzymes and transporters respond to similar signals, mainly nitrogen and stress (Tables 1 and 2). One obvious example of coordination is the induction of the proline transporters ProTs and P5CS, involved in proline transport and synthesis, respectively, in response to drought and salt (Table 2; Hare *et al.*, 1999). More subtle co-regulations probably exist and a wealth of data is available for mining in the microarray/sequencing databases to help identifying them.

### *Does amino acid transport affect metabolism?*

Supporting the fact that metabolism and transport are coordinated, a few experiments have shown that modifications of amino acid transport led to changes in amino acid content. *Arabidopsis* knockout mutants for the GABA transporter AtBAT1/GABP (Michaeli *et al.*, 2011), or the amino acid importers AtLHT1 (Hirner *et al.*, 2006), AtAAP1 (Sanders *et al.*, 2009), and AtCAT2 (Yang *et al.*, 2014*a*) accumulate amino acids at different levels compared with the wild type. Overexpression of the *GDU* genes, thought to control amino acid export, led to increased content of almost all amino acids in *Arabidopsis* (Pilot *et al.*, 2004; Pratelli *et al.*, 2010) and *Nicotiana tabaccum* (Pratelli and Pilot, 2006). Whether modification of amino acid transport has a direct effect on amino acid metabolism and amino acid content remains to be determined.

Plants respond strongly to changes in amino acid transport: knockout of the amino acid importer AtLHT1 (Liu *et al.*, 2010), overexpression of the amino acid importer CAT1 (Yang *et al.*, 2014*b*), and enhancement of amino acid export by overexpression of AtGDU1 and AtGDU3 (Chen *et al.*, 2010; Liu *et al.*, 2010) led to constitutive stress responses involving a salicylic acid response. This kind of response appears similar to the one observed after alteration of the activity of the amino acid biosynthetic pathways (see above), supporting the hypothesis of coordination of metabolism and transport activities. This effect of alteration of amino acid transport or metabolism on stress/pathogen response is intriguing and hints at a broader process in which plants could interpret alterations in amino acid homeostasis as the presence of pathogens. On the other hand, the pathogen response involves modifications of nitrogen metabolism, at least for synthesis of signalling and defence compounds (Zeier, 2013; Yang and Ludewig, 2014), making the relationship between amino acid metabolism and transport and the pathogen response more complicated. It is thus not surprising that the nitrogen status of the plant impacts on pathogen virulence, although the underlying mechanism is not understood (reviewed by Fagard *et al.*, 2014, this issue).

### *Possible mechanisms for regulation of amino acid homeostasis*

It is reasonable to speculate that some mechanisms exist that are involved in sensing the amino acid or nitrogen status of a

cell or of the plant, and that modulate metabolism, transport, and other responses accordingly. Low concentrations of Glu (50 µM) were found to affect root architecture through a sensing pathway active in the root tip, and possibly implicating auxin (Walch-Liu *et al.*, 2006). An elegant chemical genomics screening further identified a mitogen-activated protein kinase kinase kinase (MEKK1), which is involved in the Glu signal transduction (Forde *et al.*, 2013). More recently, following a large-scale correlation analysis of transcript and metabolic responses to stimuli, Hannah *et al.* (2010) postulated that Leu could play a signalling function and be involved in the expression of specific genes. The authors tested their predictions and found that many stress-response genes were induced after 90 min of exposure to 50 µM Leu (Hannah *et al.*, 2010). These results suggest that plants are able to sense external amino acids and react accordingly.

No amino acid sensor has been identified so far, but plant glutamate-like receptors (GLRs) are likely candidates for this process. GLRs have been shown to induce cation-triggered membrane depolarization upon addition of exogenous amino acids, and some knockout mutants show impaired nitrogen or carbon responses (reviewed by Forde, 2014). However it is very likely that more than one family of proteins may serve as amino acid sensors. Similar to what is suspected in mammals (Taylor, 2009), recent findings that plant transporters may be endowed with sensors properties as well (NRT1.1 for nitrate, Ho *et al.*, 2009; SULTR1;2 for sulfate, Zhang *et al.*, 2013*a*) bring a new light on nutrient sensing in plants.

The integration of nutrient sensing and the subsequent adaptation response has been described in microbes and mammals, where four main regulators, namely PII (GlnB), SnRK (SNF-1 related kinase), GCN2 (general control nonderepressible-2), and TOR (target of rapamycin), have been discovered and investigated (see below). Plants possess these metabolic regulators as well, but so far these proteins do not appear to play a critical role in the control of amino acid pathways.

In microbes, the PII protein plays a critical role in the regulation of anabolic nitrogen metabolism. It binds ATP, ADP, and 2-oxoglutarate and regulates the activity of transcription factors and metabolic enzymes (Chellamuthu *et al.*, 2013). While still present in plants, the role of PII seems limited to the control of the activity of acetyl-CoA carboxylase, the key enzyme in fatty acid synthesis, and NAGK involved in Arg metabolism in the chloroplast. PII has a local regulatory role, since its interaction with NAGK relieves the feedback inhibition from Arg in presence of 2-oxoglutarate (Chellamuthu *et al.*, 2013).

The plant SnRK proteins, similar to the AMPK and Snf1 kinases from mammals and yeast, sense sugar levels and phosphorylate numerous downstream targets including metabolic enzymes (Baena-Gonzalez *et al.*, 2007; Halford and Hey, 2009). SnRK1 appears important for linking the activity of carbohydrate and nitrogen metabolism, but no direct role in the control of amino acid pathways has been reported so far.

The pathway of GCN2 protein kinase is conserved among eukaryotes and is hypothesized to interact with the Snf1/AMPK pathways (Halford and Hey, 2009). The yeast GNC2p is an essential component of the general amino acid control mechanism (Hinnebusch, 2005). GCN2p senses high concentrations of uncharged tRNAs and prevents the synthesis of new proteins, while the yeast transcription factor GCN4 simultaneously activates more than 30 genes, many of which encode amino acid biosynthetic enzymes (Hinnebusch, 2005). A GCN2-like gene is present in *Arabidopsis*, but no gene similar to GCN4 has been found in plants (Li *et al.*, 2013), suggesting that this part of the general amino acid control mechanism is not conserved or is mediated by unknown transcription factors or other pathways in plants (Zhang *et al.*, 2008).

Finally, the TOR pathway is a major cellular regulator in mammals and yeast, coordinating cell division with nutrient availability, stress levels, and energy supply (through AMPK/Snf1) by acting on autophagy, translation, and metabolism, respectively (Wullschleger *et al.*, 2006). In mammals, glutamine transport across the membrane controls the TOR pathway, providing an input for the availability of extracellular amino acids to control autophagy (Nicklin *et al.*, 2009; Taylor, 2009). More recently, TOR was also shown to be involved in the regulation of yeast and mammalian amino acid transporters, controlling their occurrence (Matsui and Fukuda, 2013) or abundance (Rosario *et al.*, 2013) at the plasma membrane. Despite its presence in *Arabidopsis*, AtTOR may be involved in processes different from those in yeast and mammals (Zhang *et al.*, 2013*d*; Xiong and Sheen, 2014). However, links between TOR and amino acid metabolism and transport were established in a recent transcriptomics and metabolomics investigation (Caldana *et al.*, 2013). Notably, a significant increase in the levels of branched-chain, aromatic, and other amino acids (Lys, β-Ala, His, Pro, Thr, and GABA) was observed in inducible amiR-*tor* lines, as opposed to levels of Arg, ornithine, and spermidine, which were strongly reduced. Whether the increase in many amino acid levels results from *de novo* synthesis, impaired protein synthesis, autophagy (Liu and Bassham, 2010; Perez-Perez and Crespo, 2010), or altered transporter activity (possibly via ubiquitination) remains to be determined.

## Conclusion

Three decades of work aimed at elucidating amino acid metabolism in plants has led to the identification of most of the biosynthesis and catabolic pathways, and of the genes encoding the corresponding enzymes. We have a good understanding of the regulation of most of the pathways, although several unknowns remain, such as feedback inhibition in the aromatic amino acid pathway (Tzin *et al.*, 2012), or pathways that have been the subject of little recent research (e.g. Ser, Ala, Gly, Arg). We are just starting to unravel the transcriptional and post-translational regulation of the enzymes, and what we know points towards complex, multilayered regulations. Another layer of complexity will be added when transporters, their regulation mechanisms, corresponding compartmentation, and signalling pathways will be added to the model. With a much broader understanding of the

complexity of amino acid homeostasis in plants, we may be able to finally elucidate one galling observation of the 1990s (Bonner *et al.*, 1996): why can the growth inhibition by high concentrations of amino acids be suppressed by addition of Gln, and Gln only?

## Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Amino acid metabolic pathways.

Supplementary Table S1. Abbreviations of the enzymes used in Supplementary Fig. S1 and the main text.

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